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<b>(54) Title:</b> NO-MODIFIED HEMOGLOBINS AND USES THEREFOR		
<b>(57) Abstract</b>		
<p>S-nitrosohemoglobin (SNO-Hb) can be formed by reaction of Hb with S-nitrosothiol and by other methods described herein which do not result in oxidation of the heme Fe. Other methods can be used which are not specific only for thiol groups, but which nitrosate Hb more extensively, and may produce polynitrosated methb as a product or intermediate product of the method. SNO-Hb in its various forms and combinations thereof (oxy, deoxy, met; specifically S-nitrosylated, or nitrosated or nitrated to various extents) can be administered to an animal or human where it is desired to oxygenate, to scavenge free radicals, or to release NO<sup>+</sup> groups to tissues. Thiols and/or NO donating agents can also be administered to enhance the transfer of NO<sup>+</sup> groups. Examples of conditions to be treated by SNO-Hbs or other nitrosated or nitrated forms of Hb include ischemic injury, hypertension, angina, reperfusion injury and inflammation, and disorders characterized by thrombosis. Further embodiments of the invention are methods for assessing oxygen delivery to the tissues of a mammal by measuring SNO-Hb and nitrosylhemoglobin in blood.</p>		

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## NO-MODIFIED HEMOGLOBINS AND USES THEREFOR

## RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Patent Application Number 08/874,992 filed on June 12, 1997, which is a continuation-in-part of U.S. Patent Application Number 08/796,164 filed on February 6, 1997, which is a continuation-in-part of PCT/US96/14659 filed on September 13, 1996, which is a continuation of U.S. Patent Application Number 08/667,003 filed on June 20, 1996, which is a continuation-in-part of U.S. Patent Application Number 08/616,371 filed on March 15, 1996, which claims priority to U.S. Provisional Application Number 60/003,801 filed on September 15, 1995. This application is also a continuation-in-part of PCT/US96/14660 filed on September 13, 1996, which is a continuation of U.S. Patent Application Number 08/616,259 filed on March 15, 1996, which claims priority to U.S. Provisional Application Number 60/003,801 filed on September 15, 1995. The teachings of all of the above applications are each incorporated herein by reference in their entirety.



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## GOVERNMENT SUPPORT

This invention was made with government support under Grant Nos. HL52529 and HR59130 awarded by the National Institutes of Health. The government has certain rights in  
5 the invention.

## BACKGROUND OF THE INVENTION

Interactions of hemoglobin (Hb) with small diffusible ligands, such as O<sub>2</sub>, CO<sub>2</sub> and NO, are known to occur at its metal centers and amino termini. The O<sub>2</sub>/CO<sub>2</sub> delivery  
10 functions, which arise in the lung and systemic microvasculature, are allosterically controlled. Such responsiveness to the environment has not been known to apply in the case of NO. Specifically, it has been thought previously that NO does not modify the functional  
15 properties of Hb to any physiologically significant degree. Kinetic modeling predicts that the vast majority of free NO in the vasculature should be scavenged by Hb (Lancaster 1994). Accordingly, the steady-state level of NO may fall below the K<sub>m</sub> for target enzymes such as guanylate cyclase  
20 (Lancaster 1994), if not in the unperturbed organism, then with oxidant stress such as that found in atherosclerosis. These considerations raise the fundamental question of how NO exerts its biological activity.

One answer to this question is found in the propensity  
25 of nitric oxide to form S-nitrosothiols (RSNOs) (Gaston, B. et al., *Proc. Natl. Acad. Sci. USA* 90:10957-10961 (1993)), which retain NO-like vasorelaxant activity (Stamler, J.S., et al., *Proc. Natl. Acad. Sci. USA* 89:444-448 (1992)), but which can pass freely in and out of cells, unlike Hb. In  
30 particular, the NO group of RSNOs possesses nitrosonium

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(NO<sup>+</sup>) character that distinguishes it from NO itself. It is increasingly appreciated that RSNOs have the capacity to elicit certain functions that NO is incapable of (DeGroote, M.A. et al., *Proc. Natl. Acad. Sci. USA* 92:6399-6403 (1995); Stamler, J.S., *Cell* 78:931-936 (1994)). Moreover, consideration has been given to the possibility that -SNO groups in proteins serve a signaling function, perhaps analogous to phosphorylation (Stamler, J.S. et al., *Proc. Natl. Acad. Sci. USA* 89:444-448 (1992); Stamler, J.S. *Cell*, 78:931-926 (1994)). Although S-nitrosylation of proteins can regulate protein function (Stamler, J.S. et al., *Proc. Natl. Acad. Sci. USA* 89:444-448 (1992); Stamler, J.S., *Cell*, 78:931-936 (1994)), intracellular S-nitrosoproteins -- the *sine qua non* of a regulatory posttranslational modification -- has heretofore not been demonstrated.

Hemoglobin is a tetramer composed of two alpha and two beta subunits. In human Hb, each subunit contains one heme, while the beta ( $\beta$ ) subunits also contain highly reactive SH groups (cys $\beta$ 93) (Olson, J.S., *Methods in Enzymology* 76:631-651 (1981); Antonini, E. & Brunori, M. In *Hemoglobin and Myoglobin in Their Reactions with Ligands*, American Elsevier Publishing Co., Inc., New York, pp. 29-31 (1971)). These cysteine residues are highly conserved among species although their function has remained elusive.

NO (nitric oxide) is a biological "messenger molecule" which decreases blood pressure and inhibits platelet function, among other functions. NO freely diffuses from endothelium to vascular smooth muscle and platelet and across neuronal synapses to evoke biological responses. Under some conditions, reactions of NO with other components present in cells and in serum can generate toxic intermediates and products at local concentrations in tissues which are effective at inhibiting the growth of

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infectious organisms. Thus, it can be seen that a method of administering an effective concentration of NO or biologically active forms thereof would be beneficial in certain medical disorders.

5 Platelet activation is an essential component of blood coagulation and thrombotic diathesis. Activation of platelets is also seen in hematologic disorders such as sickle cell disease, in which local thrombosis is thought to be central to the painful crisis. Inhibition of  
10 platelet aggregation is therefore an important therapeutic goal in heart attacks, stroke, and shock (disseminated intravascular coagulation) and in chronic conditions such as peripheral vascular disease, heart disease, brain disease, lung disease and atherosclerosis. Researchers  
15 have attempted to give artificial hemoglobins to enhance oxygen delivery in all of the above disease states. However, as recently pointed out by Olsen and coworkers, administration of underivatized hemoglobin leads to platelet activation at sites of vascular injury (Olsen S.B.  
20 et al., *Circulation* 93:327-332 (1996)). This major problem has led experts to conclude that cell-free underivatized hemoglobins pose a significant risk of causing blood clots in the patient with vascular disease or a clotting disorder (Marcus, A.J. and J.B. Broekman, *Circulation* 93:208-209  
25 (1996)). New methods of providing for an oxygen carrier and/or a method of inhibiting platelet activation would be of benefit to patients with vascular disease or who are otherwise at risk for thrombosis.

#### SUMMARY OF THE INVENTION

30 The invention relates to methods of producing and isolating SNO-Hb (S-nitrosohemoglobin, which includes for

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instance, oxy-, deoxy-, or met- hemoglobin for use in therapy) by reaction of Hb with S-nitrosothiol in procedures which avoid oxidation of the heme. The invention also includes methods of producing isolated, nitrosated (including nitrosylated at thiols or metals) and nitrated derivatives of hemoglobins in which the heme Fe can be oxidized or not oxidized, depending on the steps of the method. The invention also relates to a method of therapy for a condition in which it is desired to oxygenate, to scavenge free radicals, or to release NO<sup>+</sup> groups or other forms of biologically active NO to tissues. A composition comprising SNO-Hb in its various forms and combinations thereof (oxy, deoxy, met; specifically S-nitrosylated, or nitrosated or nitrated to various extents) can be administered to an animal or human in these methods. Compositions comprising thiols and/or NO donating agents can also be administered to enhance the transfer of NO<sup>+</sup> groups. Examples of conditions to be treated by nitrosated or nitrated forms of hemoglobin include ischemic injury, hypertension, angina, reperfusion injury and inflammation, and diseases characterized by thrombosis. Further embodiments of the invention are methods for assessing oxygen delivery to the tissues of a mammal by measuring SNO-Hb and nitrosylhemoglobin in blood.

## 25 BRIEF DESCRIPTIONS OF THE DRAWINGS

Figures 1A-1D are spectrographs of different forms of Hb as described in Example 1.

Figure 2A is a graph showing formation, with time, of SNO-Hb by S-nitrosylation.

30 Figure 2B is a graph showing the decomposition, with time, of oxy and deoxy forms of SNO-Hb.

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Figure 3A is a graph showing the loading of red blood cells (erythrocytes) with S-nitrosocysteine, over time. The inset is a series of spectrographs of forms of Hb as described in Example 3.

5        Figure 3B is a series of tracings recording isometric tone of a rabbit aortic ring following treatment of the aortic ring with various agents as described in Example 3.

Figure 4A is a graph of change in tension of a rabbit aortic ring versus concentration of the Hb used in the  
10    experiment.

Figure 4B is a graph of change in tension of a rabbit aortic ring versus concentration of the Hb used in the experiment, where glutathione was also added to test the effect as compared to Figure 4A.

15        Figure 4C is a graph of the ratio of S-nitrosoglutathione formed/starting SNO-Hb concentration versus time, showing rates of NO group transfer from oxy and met forms of Hb to glutathione.

Figure 4D is a graph of S-nitrosothiols exported from  
20    loaded red blood cells over time.

Figure 5 is a graph showing the mean arterial blood pressure in rats after they received various doses of oxyHb ( $\blacktriangle$ ), SNO-oxyHb ( $\blacksquare$ ), or SNO-metHb ( $\bullet$ ).

Figures 6A-6F are a series of tracings recording blood  
25    pressure (Figures 6A and 6B), coronary artery diameter (Figures 6C and 6D) and coronary artery flow (Figures 6E and 6F), after administration of S-nitrosohemoglobin to anesthetized dogs.

Figure 7A is a graph illustrating the effect of  
30    unmodified HbA<sub>0</sub> on platelet aggregation. The maximal extent of aggregation of platelets is plotted against the concentration of HbA (10 nM to 100  $\mu$ M) preincubated with platelets. Experiments were performed as in Example 9.

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Vertical bars plotted with each data point indicate the standard deviation.

Figure 7B is a graph illustrating the effect of S-nitroso(oxy)hemoglobin on platelet aggregation. The  
5 normalized maximal extent of aggregation of platelets is plotted against the concentration of HbA (10 nM to 100  $\mu$ M) preincubated with platelets.

Figure 7C is a graph illustrating the antiaggregation effects on platelets by S-nitroso(met)hemoglobin.

10 Figure 8 is a bar graph showing the amount of cGMP (guanosine 3',5'-cyclic phosphoric acid), assayed as in Example 10, for 1, 10 and 100  $\mu$ M concentrations of native Hb, SNO-oxyHb or SNO-metHb interacting with  $10^8$  platelets.

Figure 9A is a graph which shows the spectra  
15 (absorbance versus wavelength in nanometers) of HbA<sub>0</sub> treated as described in Example 11. The shift in the wavelength of maximum absorbance of spectrum B relative to spectrum A illustrates the extent of addition of NO groups to HbA<sub>0</sub>.

20 Figure 9B is a graph which shows the spectra of Hb treated with 100-fold excess S-nitrosoglutathione as described in Example 11.

Figure 9C is a graph which shows the spectra of HbA<sub>0</sub> treated with excess S-nitrosocysteine as described in  
25 Example 11.

Figure 9D is a graph which shows the spectra of rat Hb treated with 100-fold excess S-nitrosocysteine. Spectrum A shows nitrosated Hb not further treated with dithionite; spectrum B shows nitrosated Hb further treated with  
30 dithionite.

Figure 9E is a graph illustrating the increase in nitrosated Hb product with time by reacting HbA<sub>0</sub> with either 100x excess S-nitrosocysteine (top curve) or 10x

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excess S-nitrosocysteine (middle curve). HbA<sub>0</sub> was preincubated with 100  $\mu$ M inositol hexaphosphate before reacting with 10x excess S-nitrosocysteine (bottom curve; triangle points). (See Example 11.)

5        Figure 10 is a graph illustrating the percent change, with time, in blood flow measured in caudatoputamen nucleus of rats after injection of the rats with:  $\circ$ , 100 nmol/kg SNO-Hb;  $\bullet$ , 1000 nmol/kg SNO-Hb; or  $\blacksquare$ , 1000 nmol/kg underivatized Hb (see Example 12).

10        Figure 11 is a graph illustrating the percent change in tension of a ring of aorta from rabbit, plotted as a function of the log of the molar concentration of hemoglobin tested (see Example 13).  $\bullet$ , Hb treated with S-nitrosocysteine at a ratio of 1:1 CYSNO/Hb;  $\circ$ , Hb treated  
15        with CYSNO at a ratio of 10:1 CYSNO/Hb;  $\blacklozenge$ , Hb treated with CYSNO at a ratio of 100:1.

Figure 12 is a graph of the absorbance versus the wavelength of light (nm), for aqueous solutions of 17  $\mu$ M deoxyhemoglobin, 1  $\mu$ M NO, and varying amounts of dissolved  
20        oxygen added by sequential injections of room air. The absorbance of the initial solution (no added air) is shown by the curve with the highest peak at approximately 430 nm. Sequential additions of 50  $\mu$ l of air shift the curve leftwards on the graph. See Example 14.

25        Figure 13 is a graph showing the yield of SNO-Hb as micromolar concentration (left axis, diamonds) and as % of NO added (right axis, squares), plotted against the heme:NO ratio, when nitrosyl-deoxyHb made at various ratios of heme:NO was exposed to oxygen. See Example 15.

30        Figure 14A is a graph showing difference spectra (each a spectrum of the NO and Hb mixture minus spectrum of the starting deoxyHb), for 17  $\mu$ M hemoglobin and NO

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mixtures, for the concentrations of NO shown. See Example 16.

Figure 14B is a graph showing the peak wavelength of the difference spectra plotted against the concentration of nitric oxide added to the solution as in Figure 14B.

Figure 15A is a graph showing difference spectra (deoxyhemoglobin and air mixtures minus initial deoxyhemoglobin spectrum), for successive additions of air.

Figure 15B is a graph showing difference spectra (20  $\mu\text{M}$  deoxyhemoglobin and 1  $\mu\text{M}$  NO mixture, with successive additions of air, minus initial deoxyhemoglobin spectrum). See Example 17.

Figure 16 is a graph showing two difference spectra ( $A_{418}$  of hemoglobin and NO solution at heme:NO 20:1 minus initial deoxyhemoglobin  $A_{418}$ ) for the mutant  $\beta 93\text{Ala}$  Hb and wild type  $\beta 93\text{Cys}$  Hb. See Example 18.

Figure 17 is a graph showing the yield of SNO-Hb as micromolar concentration (left axis, diamonds) and as % of NO added (right axis, squares), plotted against the heme:NO ratio, when nitrosyl-deoxyHb made at various ratios of heme:NO was exposed to oxygen. See Example 19.

Figure 18A is a graph showing the percentage content of oxidized hemoglobin (metHb) for different concentrations of Hb (symbols below) to which NO was added to reach varying final concentrations (horizontal axis).

◆ represents 1.26  $\mu\text{M}$  hemoglobin, ■ represents 5.6  $\mu\text{M}$  hemoglobin, ▲ represents 7.0  $\mu\text{M}$  hemoglobin, X represents 10.3  $\mu\text{M}$  hemoglobin, ✕ represents 13.3  $\mu\text{M}$  hemoglobin, and • represents 18.3  $\mu\text{M}$  hemoglobin. See Example 20.

Figure 18B is a graph showing the yield of oxidized hemoglobin ( $\mu\text{M}$ ) plotted against the final concentration of NO added to solutions of Hb at the concentrations indicated by the symbols as for Figure 18A.



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Figure 19 is a graph showing the concentration of oxidized Hb (methHb) plotted against the NO concentration, in experiments performed as described in Example 21 in 10 mM ( $\blacklozenge$ ), 100 mM ( $\blacktriangle$ ), or 1 M ( $\times$ ) sodium phosphate buffer, pH 7.4.

Figures 20A and 20B are graphs showing the contractile effects of oxyHb, SNO-oxyHb, deoxy-Hb and SNO-deoxy-Hb on thoracic aortic ring isolated from rabbit. Measurements are percent increase in tension of aortic ring as a function of the log of the concentration of hemoglobin or SNO-hemoglobin. Measurements are made after the tension has stabilized.

Figure 20C is a graph showing the percent change in tension of contracted aortic ring as a function of the log concentration of SNO-hemoglobin at the concentrations of  $O_2$  indicated, in addition to 10  $\mu$ M glutathione.

Figure 20D is a graph showing the percent change in tension of contracted aortic ring as a function of the log concentration of SNO-glutathione, in the concentrations of  $O_2$  indicated.

Figure 21A and Figure 21B are each a series of four graphs illustrating the change with time in tension of rabbit aortic ring upon the addition of red blood cells treated with S-nitrosocysteine ("red blood cells loaded with nitric oxide"), or untreated red blood cells, as indicated, in the concentration of  $O_2$  indicated. Figure 21C is a graph illustrating the change with time in tension of rabbit aortic ring contracted with phenylephrine under hypoxic conditions (6-7 torr) and then exposed to either 1  $\mu$ M Hb or SNO-Hb.

Figure 22 is a bar graph depicting the concentrations of FeNO/Hb and SNO/Hb in venous or arterial blood as

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measured in Example 24. ATA=atmospheres of absolute pressure.

- Figures 23A-23I are each a graph showing the effects of SNO-Hb (●) and Hb (■) (1  $\mu$ mol/kg infused over 3 minutes) on local blood flow in substantia nigra (SN), caudate putamen nucleus, and parietal cortex of rats, in 21% O<sub>2</sub> (Figures 23A, 23B and 23C), in 100% O<sub>2</sub> (Figures 23D, 23E and 23F), and in 100% O<sub>2</sub> at 3 atmospheres absolute pressure (Figures 23G, 23H and 23I) as measured in Example 25.
- Figure 24A is a bar graph showing the percent change in blood pressure of rats, during exposure to three different conditions (inspired O<sub>2</sub> concentrations of 21%, 100%, or 100% O<sub>2</sub> at 3 ATA) upon infusion of GSNO, SNO-Hb, or Hb, as tested in Example 26.
- Figure 24B is a bar graph showing the percent change in blood pressure of rats [pre-administered (+ L-NMMA), or not preadministered (- L-NMMA), N<sup>G</sup>-monomethyl-L-arginine] upon infusion of SNO-RBCs (RBCs = red blood cells), as tested in Example 26.
- Figure 25 is a bar graph showing the results of photolysis-chemiluminescence assays to measure NO bound in the form of S-nitrosothiol and NO bound at the heme, on SNO-Hb(FeII)<sub>2</sub> and deoxyHb(FeII)NO prepared as described in Example 27.
- Figure 26 is a diagram illustrating (upper panel) alternative reactions proposed for  $\beta$ -chain nitrosyl hemes in the T structure and (lower panel) a model of NO binding to hemes and thiols of hemoglobin, in the circulation of a mammal or bird, for example.

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## DETAILED DESCRIPTION OF THE INVENTION

Roles for Hemoglobin in Physiology

The increase in SNO-Hb content of red cells across the pulmonary circuit (right ventricular inport-left ventricle) suggests that the Hb molecule is S-nitrosylated in the lung. Selective transfer of the NO group from endogenous RSNOs found in lung (Gaston, et al. (1993)) and blood (Scharfstein, J.S. et al., *J. Clin. Invest.* 94:1432-1439 (1995)) to SH groups of Hb, substantiate these findings.

The corresponding decline in Hb(FeII)NO levels across the pulmonary bed reveals a role for the lung either in the elimination of NO or in its intramolecular transfer from heme to cys $\beta$ 93. Taken in aggregate, these data extend the list of function-regulating interactions of Hb with small molecules within the respiratory system, previously known to include the elimination of CO and CO<sub>2</sub>, and uptake of O<sub>2</sub>. Since, as demonstrated herein, oxygenation of Hb leads to structural changes that increase the NO-related reactivity of cys $\beta$ 93, O<sub>2</sub> can now be regarded as an allosteric effector of Hb S-nitrosylation.

The arterial-venous difference in SNO-Hb concentration suggests that the protein acts as an NO group donor in the systemic circulation. There is good indication that SNO-Hb functions in regulation of vasomotor tone. In the microcirculation, where control of blood pressure is achieved, erythrocytes come in intimate contact with endothelial surfaces. Under these conditions, Hb can contract the vasculature by sharply decreasing the steady state level of free NO (Lancaster, J.R., (1994)). This is believed to contribute to the increases in blood pressure that occur with infusion of cell-free Hbs (Vogel, W.M., et

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al., *Am. J. Physiol.*, 251:H413-H420 (1986); Olsen, S.B., et al., *Circulation* 93:329-332 (1996)). The transient nature of such hypertensive responses, however, is consistent with the subsequent formation of SNO-Hb which counteracts this effect, evidenced by its lowering of blood pressure at naturally occurring concentrations. Thus, the capacity of the erythrocyte to support the synthesis and metabolism of SNO-Hb is important for normal blood flow.

Mammals must have adopted unique molecular mechanisms to ensure adequate NO delivery in the microcirculation. Results herein suggest that Hb has evolved both electronic and conformational switching mechanisms to achieve NO homeostasis. Specifically, NO scavenging by the metal center(s) of SNO-Hb(FeII)O<sub>2</sub> is sensed through its conversion to met(FeIII) (Figure 1B). This electronic switch effects decomposition of SNO-Hb with NO group release (Figures 3A, 3B, 4A). In this manner, the NO-related activity of SNO-Hb is partly determined by the amount of NO scavenged. Changes in O<sub>2</sub> tension also function to regulate NO delivery, as it is observed herein that NO release is facilitated by deoxygenation. This allosteric effect promotes the efficient utilization of O<sub>2</sub>, as NO controls mitochondrial respiration (Shen, W., et al., *Circulation* 92:3505-3512 (1995)).

S-nitrosothiol groups in proteins have been implicated in NO metabolism and in regulation of cellular functions (Stamler, J.S., et al., *Proc. Natl. Acad. Sci. USA* 89:444-448 (1992); Stamler, J.S., *Cell* 78:931-936 (1994)). The identification of SNO-Hb in erythrocytes is the first demonstration of an intracellular S-nitrosoprotein and gives further credence to the role of such proteins in cellular regulation. The question arises as to how SNO-Hb relaxes blood vessels when any free NO released would be

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scavenged instantaneously by Hb itself according to previous theories (Lancaster, J.R., (1994)). Noteworthy in this regard are studies showing that RSNO activity involves nitrosyl (NO<sup>+</sup>) transfer to thiol acceptors (Scharfstein, J.S., et al., (1994); Arnette, D.R. and Stamler, J.S., *Arch. Biochem. Biophys.* 318:279-285 (1995); Stamler, J.S., et al., *Proc. Natl. Acad. Sci. USA* 89:7674-7677 (1992)), which serve to protect the NO-related activity from inactivation at metal centers. Findings presented herein indicate that S-nitrosothiol/thiol exchange with glutathione (forming GSNO) occurs within erythrocytes, and is influenced by the oxidation state of heme and its occupation by ligand. Certain activities of GSNO in bacteria require transport of intact dipeptide (i.e., S-nitrosocysteinyglycine) across the cell membrane (DeGroot, M.A., et al., *Proc. Natl. Acad. Sci. USA* 92:6399-6403 (1995)). The data presented below in the Examples show that S-nitrosothiol transport occurs also in eukaryotic cells. GSNO, or related thiol carriers exported by erythrocytes (Kondo, T., et al., *Methods in Enzymology*, Packer, L., ed., Academic Press, 252:72-83 (1995)), might also initiate signaling in or at the plasmalemma (Stamler, J.S., *Cell* 78:931-936 (1994)), given reports of thiol-dependent activation of potassium channels by EDRF (Bolotina, V.M., et al., *Nature* 368:850-853 (1994)). Alternative possibilities also merit consideration. In particular, reports that Hb associates with red cell membranes via cys $\beta$ 93 (Salhany, J.M. and Gaines, K.C., *Trends in Biochem. Sci.*, pp. 13-15 (Jan. 1981)) places Hb in a position to donate the NO group directly to contacting endothelial surfaces, perhaps via SNO/SH exchange. Cell surface interactions appear to be operative in signaling mediated by other S-nitrosoproteins (Stamler, J.S., et al.,

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Proc. Natl. Acad. Sci. USA, 89:444-448 (1992); Stamler, J.S., Cell, 78:931-936 (1994)).

The highly conserved Cys $\beta$ 93 residues in Hb influence the oxygen affinity and redox potential of the heme iron and its physiochemical properties (Garel, C., et al., Biochem. 123:513-519 (1982); Jocelyn, P.C., et al., Biochemistry of the SH Group, p. 243, Academic Press, London; (1972); Craescu, C.T., J. Biol. Chem. 261:14710-14716 (1986); Mansouri, A., Biochem. Biophys. Res. Commun., 89:441-447 (1979)). Nonetheless, their long sought-after physiological function has remained a mystery. The studies herein suggest new sensory and regulatory roles for Hb, in which Cys $\beta$ 93 functions in transducing NO-related signals to the vessel wall. In particular, the physiological function of Cys $\beta$ 93, which is invariant in all mammals and birds, is to deliver under allosteric control, NO-related biological activity that cannot be scavenged by heme. Thus, these data bring to light a dynamic circuit for the NO group in which intraerythrocytic Hb participates as both a sink and a donor, depending on its microenvironment. Such observations provide answers to paradoxes that arise from conceptual frameworks based solely on diffusional spread and reaction of free NO (Lancaster, J.R., (1994); Wood and Garthwaite, J. Neuropharmacology 33:1235-1244 (1994)); and has implications that extend to other thiol- and metal-containing (heme) proteins, such as nitric oxide synthase and guanylate cyclase.

The discoveries reported here have direct therapeutic implications. Specifically, concerns over loss of NO-related activity due to inactivation by blood Hb (Lancaster, J.R., (1994)) are obviated by the presence of an RSNO subject to allosteric control. Forms of SNO-Hb can be free of the adverse hypertensive properties of cell-free

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Hb preparations that result from NO scavenging at the metal centers. A composition comprising one or more of the various forms of cell-free SNO-Hb (e.g., SNO-Hb[FeII]O<sub>2</sub>, SNO-Hb[FeIII], SNO-Hb[FeII]CO) can be administered in a pharmaceutically acceptable vehicle to a human or other mammal to act as a blood substitute.

Blood Flow Regulation by S-Nitrosohemoglobin is Controlled by the Physiological Oxygen Gradient

In the classical allosteric model, Hb exists in two alternative structures, named R (for relaxed, high O<sub>2</sub> affinity) and T (for tense, low O<sub>2</sub> affinity). The rapid transit time of blood through the capillaries requires that Hb assume the T-structure to efficiently deliver O<sub>2</sub> (M.F. Perutz, pp. 127-178 in *Molecular Basis of Blood Diseases*, G. Stamatayanopoulos, Ed. (W.B. Saunders Co., Philadelphia, 1987); Voet, D. and Voet, J.G., pp. 215-235 (John Wiley & Sons Inc., New York, 1995). The switch from R to T in red blood cells normally takes place when the second molecule of O<sub>2</sub> is liberated. This allosteric transition also controls the reactivity of two highly conserved cysteine<sup>93</sup> residues that can react with 'NO'. Thiol affinity for NO is high in the R or oxy structure and low in T or deoxy structure. This means that the NO group is released from thiols of Hb in low PO<sub>2</sub> and explains the arterial-venous (A-V) difference in the S-nitrosohemoglobin (SNO-Hb) level of blood (see Table 2, Example 8). A major function of (S)NO in the vasculature is to regulate blood flow, which is controlled by the resistance arterioles (Guyton, A.C., in *Textbook of Medical Physiology* (W.B. Saunders Co., Philadelphia, 1981) pp. 504-513). It is shown from the Examples herein that (partial) deoxygenation of SNO-Hb in these vessels (Duling, B. and Berne, R.M.

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- Circulation Research*, 27:669 (1970); Popel, A.S., et al., (erratum *Am. J. Physiol.* 26(3) pt. 2). *Am. J. Physiol.* 256, H921 (1989); Swain, D.P. and Pittman, R.N. *Am. J. Physiol.* 256, H247-H255 (1989); Torres, I. et al.,
- 5 *Microvasc. Res.*, 51:202-212 (1996); Buerk, D. et al., *Microvasc. Res.*, 45:134-148 (1993)) actually promotes O<sub>2</sub> delivery by liberating (S)NO. That is, the allosteric transition in Hb functions to release (S)NO in order to increase blood flow.
- 10 O<sub>2</sub> delivery to tissues is a function of the O<sub>2</sub> content of blood and blood flow (Dewhirst, M.W. et al., *Cancer Res.*, 54:3333-3336 (1994); Kerger, H. et al., *Am. J. Physiol.*, 268:H802-H810 (1995)). Blood oxygen content is largely determined by Hb, which undergoes allosteric
- 15 transitions in the lung and systemic microvasculature that promote the binding and release of O<sub>2</sub> (L. Stryer, in *Biochemistry* L. Stryer, Ed. (W.H. Freeman & Co., San Francisco, 1981) pp. 43-82; Guyton, A.C. in *Textbook of Medical Physiology* (W.B. Saunders Co., Philadelphia, 1981);
- 20 Perutz, M.F., pp. 127-178 in *Molecular Basis of Blood Diseases*, G. Stamatayanopoulos, Ed. (W.B. Saunders Co., Philadelphia, 1987); Voet, D. and Voet, J.G. (John Wiley & Sons Inc., New York, 1995) pp. 215-235 pp. 208-215, 224-225, 230-245, 344-355)). Intimate contact between
- 25 erythrocyte and endothelium is believed to facilitate O<sub>2</sub> delivery by minimizing the distance for O<sub>2</sub> diffusion into surrounding tissues (Caro, C.G. et al., Oxford University Press, Oxford, 363 (1978)). On the other hand, regional blood flow is regulated by metabolic requirements of the
- 30 tissue: blood flow is increased by hypoxia and decreased when O<sub>2</sub> supply exceeds demand (Guyton, A.C., in *Textbook of Medical Physiology* (W.B. Saunders Co., Philadelphia, 1981) pp. 504-513)). These classical physiological responses are



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thought to be partly mediated by changes in the level of endothelial-derived NO and its biological equivalents (Park, K.H. et al., *Circ. Res.*, 71:992-1001 (1992); Hampl, V. et al., *J. Appl. Physiol.* 75(4):1748-1757 (1993)).

- 5        This standard picture has its problems. First, it is puzzling that significant O<sub>2</sub> exchange occurs in the precapillary resistance vessels (evidenced by the periarteriolar O<sub>2</sub> gradient; Duling, B. and Berne, R.M. *Circulation Research*, 27:669 (1970); Popel, A.S., et al.,  
10 (erratum *Am. J. Physiol.* 26(3) pt. 2). *Am. J. Physiol.* 256, H921 (1989); Swain, D.P. and Pittman, R.N. *Am. J. Physiol.* 256, H247-H255 (1989); Torres, I. et al., *Microvasc. Res.*, 51:202-212 (1996); Buerk, D. et al., *Microvasc. Res.*, 45:134-148 (1993)). Why is O<sub>2</sub> lost to  
15 counter-current venous exchange prior to reaching the tissues? Second, close contact between endothelial surfaces and erythrocytes leads to sequestration of NO by Hb (Stamler, J.S., *Nature*, 380:108-111 (1996); Perutz, M.F., *Nature*, 380:205-206 (1996)). Decreases in the  
20 steady-state levels of NO in terminal arterioles (King, C.E. et al., *J. Appl. Physiol.*, 76(3):1166-1171 (1994); Shen, W. et al., *Circulation*, 92:3505-3512 (1995); Kobzik, L. et al., *Biochem. Biophys. Res. Comm.*, 211(2):375-381 (1995); Persson, M.G., et al., *Br. J. Pharmacol.*, 100:463-  
25 466 (1990) and capillaries (Mitchell, D., and Tyml, K., *Am. J. Physiol.*, 270 *Heart Circ. Physiol.*, 39), H1696-H1703 (1996)) contract blood vessels, blunt hypoxic vasodilation and reduce red cell velocity. This line of reasoning leads to the paradox: the red blood cell seems to oppose its own  
30 O<sub>2</sub> delivery function (note in vivo effects of Hb in Figures 10A-10I).

The finding that the O<sub>2</sub> gradient in precapillary resistance vessels promotes NO group release from SNO-Hb

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appears to solve these problems. SNO-Hb compensates for NO scavenging at the heme iron by assuming the T-structure which liberates SNO. Specifically, Cys93 donates the NO group in deoxy structure whereas it cannot do so in the oxy conformation. Accordingly, the O<sub>2</sub> gradient determines whether SNO-Hb dilates or constricts blood vessels. Stated another way, SNO-Hb senses the tissue PO<sub>2</sub> (i.e., the periarteriolar O<sub>2</sub> gradient) and then utilizes the allosteric transition as a means to control arteriolar tone. If the tissue is hypoxic (i.e., the O<sub>2</sub> gradient is high), SNO is released to improve blood flow. However, if O<sub>2</sub> supply exceeds demand (i.e., the O<sub>2</sub> gradient is small), SNO-Hb holds on to the NO group by maintaining the R-structure--with the net effect of reducing blood flow in line with demand. SNO-Hb thereby contributes to the classical physiological responses of hypoxic vasodilation and hyperoxic vasoconstriction.

Based on studies described herein, especially Examples 22-26, the following picture emerges. Partially nitrosylated Hb (Hb[FeII]NO) enters the lung in T-structure (see venous measurements in Figure 22). There, S-nitrosylation is facilitated by the O<sub>2</sub>-induced conformational change in Hb. SNO-oxyHb (SNO-Hb[FeII]O<sub>2</sub>) enters the systemic circulation in R-structure (see arterial levels in Figure 22). Oxygen losses in precapillary resistance vessels then effect an allosteric transition (from R to T) in Hb which liberates 'NO' to dilate blood vessels (see especially Figures 20D and 23A-C). NO released from Hb is transferred directly to the endothelium, or by way of low mass S-nitrosothiols--such as GSNO--which are exported from RBCs (see Figure 4D and Example 4; see also Figures 20C and 24A). Thus, the O<sub>2</sub> gradient in arterioles serves to enhance O<sub>2</sub> delivery: it

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promotes an allosteric transition in Hb which releases NO-related activity to improve blood flow.

#### Assay methods

The invention also relates to a method for determining  
5 the concentration of nitrosyl(FeII)- hemoglobin in a blood sample, thereby serving as a measure of the level of NO in the animal or human from which the blood sample has been taken. The method is related to one used previously for the measurement of S-nitrosoproteins and smaller molecular  
10 weight S-nitrosothiols in plasma (See U.S. Patent No. 5,459,076; Oct. 17, 1995. The contents of this patent are hereby incorporated by reference in their entirety.) However, the primary focus of the present invention is on assaying for nitrosyl(FeII)-hemoglobin rather than S-  
15 nitrosothiols.

In contrast to the previous method, in which the red blood cells were removed and discarded from the sample to be analyzed, the subject invention method uses the red blood cells. The method measures NO which has reacted with  
20 the thiol groups of hemoglobin in the form of S-nitroso-hemoglobin (SNO-Hb) as well as NO bound to the Fe of the heme (nitrosyl(FeII)-hemoglobin or Hb(FeII)NO). As shown in the table, the level of S-nitroso-hemoglobin in venous blood is negligible compared to the level of Hb(FeII)NO.  
25 Therefore, to specifically measure the level of Hb(FeII)NO in venous blood, it is unnecessary to include steps in which Hb samples are divided into two aliquots which are then either treated or not treated with a 10-fold excess of HgCl<sub>2</sub> over the protein concentration. Reaction of Hb with  
30 HgCl<sub>2</sub> removes NO from thiol groups selectively, without disturbing NO bound at the heme. Values for NO obtained from the HgCl<sub>2</sub> reaction, if significant, should be

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subtracted from the total NO obtained for the measurements without the  $\text{HgCl}_2$  reaction, to obtain an accurate value for  $\text{Hb(FeII)NO}$ .

In one embodiment of the invention, a blood sample is taken from a mammal, such as a human, and the solid parts including cells are isolated away from the remaining fluid. The cells are then lysed by standard methods, and a protein fraction is prepared from the lysate. Before quantitating nitric oxide adducts (nitrosonium adducts, which include low molecular weight S-nitrosothiols (which are small enough to be freely diffusible through cell membranes, such as the S-nitrosothiol S-nitrosoglutathione) and high molecular weight S-nitrosothiols such as S-nitroso-proteins), it is preferable to first remove low molecular weight S-nitrosothiols endogenous to the red blood cells, which would also contribute to the NO value, by a step which separates low molecular weight molecules away from the red blood cell proteins (referred to as desalting). This step can include, for example, dialysis or column chromatography based on separation by size of the molecules. A further step is to subject the protein fraction to photolysis, as in a photolysis cell, where it is irradiated with light of the appropriate wavelength to liberate NO from the various forms of hemoglobin. The resulting NO is detected by reaction with ozone.

One embodiment of the invention utilizes a chemiluminescence apparatus in which a photolysis cell is linked directly to the reaction chamber and detector portion, thereby bypassing the pyrolyzer. A sample of the blood protein fraction is injected into the photolysis cell, either directly, or as chromatographic effluent from a high-performance liquid or gas chromatography system which is connected to the photolysis cell.

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The sample is then irradiated with a mercury vapor lamp, and directed through a series of cold traps, where liquid and gaseous fractions which are less volatile than nitric oxide (such as nitrite and nitrate) are eliminated, leaving only free nitric oxide remaining in the cell. The nitric oxide is then transported by a gaseous stream, preferably helium, into the chemiluminescence spectrometer. In the alternative, other inert gases may be used.

Once present in the chemiluminescence spectrometer, the free nitric oxide is detected by its chemical reaction with ozone, resulting in the generation of signals that are recorded on a digital integrator. If desired, flow rates and illumination levels in the photolysis cell can be adjusted to cause complete photolysis of the S-nitric oxide bond of the S-nitrosothiol compounds. Flow rates and illumination levels may be adjusted by routine methods available in the art, in order to achieve optimal cleavage of the bond between the particular adduct and nitric oxide, nitrosonium or nitroxyl, whichever is bound.

In a variation, the invention relates to a method for detecting S-nitrosothiols, including primarily S-nitroso-hemoglobin (SNO-Hb) in a blood sample. This method comprises inactivating the chemiluminescence, signal-generating capability of any nitric oxide which is associated with a thiol, in the protein fraction derived from the blood sample, and determining the amount of thiol-bound nitric oxide by measuring the quantitative difference between total nitric oxide and nitric oxide remaining after inactivation.

A particular embodiment of this variation relates to a method in which the protein fraction derived from the blood sample is treated with a source of mercury ions, mercurous ions being preferred, followed by air incubation, which

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- oxidizes the nitric oxide and nitrosonium and renders them undetectable. Compounds suitable for pretreatment include  $\text{Hg}_2\text{Cl}_2$  and other mercurous ion salts and organic mercurials. The treated sample is then injected into the
- 5 photolysis cell, where  $\text{NO}^+$  is converted to  $\text{NO}^\cdot$  (nitric oxide) and the nitric oxide is detected by the chemiluminescence method described above. The amount of nitric oxide which is specifically derived from S-nitrosothiols is determined by comparing the
- 10 chemiluminescence signal generated by the mercury ion-treated sample, with a chemiluminescence signal generated by a sample of the equivalent biological fluid which is not treated with mercury ion prior to injection into the photolysis cell.
- 15 In a further embodiment of the claimed invention, the methods described herein can be utilized to determine the presence of a disease state which involves abnormal levels of nitric oxide and its biologically active equivalents, by monitoring  $\text{Hb(FeII)NO}$  and  $\text{SNO-Hb}$  levels in blood, and more
- 20 particularly,  $\text{Hb(FeII)NO}$  in venous blood from a patient. The ability to specifically assay for  $\text{Hb(FeII)NO}$  in venous blood distinguishes this assay over previously known methods. Nitric oxide adducts represent a pool of bioactive nitric oxide in physiological systems.
- 25 Therefore, in disease states in which the pathogenesis derives from the effects of abnormal levels of nitric oxide, these methods provide a means for the clinician to determine the presence of, and monitor the extent of, the disease state. Such information enables the clinician to
- 30 determine the appropriate pharmacological intervention necessary to treat the disease state. Such disease states and medical disorders include, but are not limited to, respiratory distress, septic shock, cardiogenic shock,

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hypovolemic shock, atherosclerosis, hyperhomocysteinemia, venous thrombosis, arterial thrombosis, coronary occlusion, pulmonary embolism, cerebrovascular accidents, vascular fibrosis, ectopia lentis, osteoporosis, mental retardation, skeletal deformities, pulmonary hypertension, malignancy, infections, inflammation, asthma, tolerance to narcotics and central nervous system disorders. Furthermore, the use of these methods is not limited to these diseases. This method can be of use in assaying biologically active nitric oxide equivalents in any disease state or medical disorder in which nitric oxide is implicated.

The data set forth in the Examples below demonstrate that a determination of NO bound to hemoglobin as nitrosylhemoglobin and SNO-Hb can be used to assess the efficiency of oxygen delivery to the tissues of an animal or a human patient. Values for nitrosylhemoglobin and SNO-Hb in blood can be determined together, in one method, or they can be determined in separate methods. An additional determination for oxygen in the blood, as measured by methods known in the art, can be used in conjunction with determinations of nitrosylhemoglobin and SNO-Hb concentrations, to assess oxygen delivery to a site in the body of a human or other mammal from which a blood sample is taken.

#### 25 Further embodiments

The subject invention relates to a method of loading cells with a nitrosating agent as exemplified for red blood cells as in Figure 3A, but which can be accomplished in more ways. Suitable conditions for pH and for the temperature of incubation are, for example, a range of pH 7-9, with pH 8 being preferred, and a temperature range of 25 to 37°C. For red blood cells, short incubation times of

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1 to 3 minutes are preferred for limiting the formation of S-nitrosylated forms of Hb. However, intracellular concentrations of 1 mM nitrosating agent can be reached.

The nitrosating agent should be a good donor of NO<sup>+</sup> and should be able to diffuse through the cell membrane of the target cell type. That is, it is preferably of low molecular weight, compared to the molecular weight of S-nitrosoproteins. Examples are S-nitroso-N-acetylcysteine, S-nitrosocysteinyglycine, S-nitrosocysteine, S-nitrosohomocysteine, organic nitrates and nitrites, metal nitrosyl complexes, S-nitro and S-nitroso compounds, thionitrites, diazeniumdiolates, and other related nitrosating agents as defined in Feelisch, M. and Stamler, J.S., "Donors of Nitrogen Oxides" chapter 7, pp. 71-115 In Methods in Nitric Oxide Research (Feelisch, M. and Stamler, J.S., eds.) John Wiley and Sons, Ltd., Chichester, U.K. (1996), the contents of which chapter are hereby incorporated by reference in their entirety. Nitrosating agents have differential activities for different reactive groups on metal-containing proteins. A nitrosating agent can be chosen for minimal oxidation of the heme iron of Hb, and maximum activity in nitrosylating thiol groups such as found on cysteine. Assay methods are available for detection of nitrosation products, including S-nitrosothiols. See Stamler et al., U.S. Patent Number 5,459,076, the contents of which are hereby incorporated by reference in their entirety. See also, for example, Keefer, L.K., and Williams, D. L. H., "Detection of Nitric Oxide Via its Derived Nitrosation Products," chapter 35, pp. 509-519 In Methods in Nitric Oxide Research (Feelisch, M. and Stamler, J.S., eds.) John Wiley and Sons, Ltd., Chichester, U.K., 1996; see also Stamler, J.S. and Feelisch, M., "Preparation and Detection of S-



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Nitrosothiols," chapter 36, pp. 521-539, *ibid.* Nitrite and nitrate products can be assayed by methods described, for instance, in Schmidt, H.H.H.W. and Kelm, M., "Determination of Nitrite and Nitrate by the Griess Reaction," chapter 33, 5 pp. 491-497, *ibid.*, and in Leone, A.M. and Kelm, M., "Capillary Electrophoretic and Liquid Chromatographic Analysis of Nitrite and Nitrate," chapter 34, pp. 499-507, *ibid.*

Such low molecular weight nitrosating agents can be 10 used in red blood cells to deliver NO-related activity to tissues. Treatment of red blood cells with nitrosating agent further serves to increase the O<sub>2</sub> delivery capacity of red blood cells. Such treatment of red blood cells also allows for the scavenging of free radicals, such as oxygen 15 free radicals, throughout the circulation. It is possible to load red blood cells with S-nitrosothiol, for example, by a process of removing whole blood from a patient's body (as a minimal method of isolating red blood cells), treating the red blood cells with low molecular weight 20 nitrosating agent, such as by incubating the red blood cells in a solution of S-nitrosothiol, and then reintroducing the red blood cells into the same patient, thereby allowing the treatment of a number of types of diseases and medical disorders, such as those which are 25 characterized by abnormal O<sub>2</sub> metabolism of tissues, oxygen-related toxicity, abnormal vascular tone, abnormal red blood cell adhesion, and/or abnormal O<sub>2</sub> delivery by red blood cells. Such diseases can include, but are not limited to, ischemic injury, hypertension, shock, angina, 30 stroke, reperfusion injury, acute lung injury, sickle cell anemia, and blood borne infectious diseases such as schistosomiasis and malaria. The use of such "loaded" red blood cells also extends to blood substitute therapy and to

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the preservation of living organs, such as organs for transplantation. In some cases, it will be appropriate to treat a patient with loaded red blood cells originating from a different person.

5       A particular illustration of the mechanism of the treatment method is presented here by considering sickle cell anemia. Sickle cell patients suffer from frequent vascular occlusive crises which manifest in clinical syndromes such as the acute chest syndrome and hepatic  
10   dysfunction. Both endothelial cell dysfunction, resulting in a clotting diathesis as well as dysfunction intrinsic to the red blood cell, are central to disease pathogenesis. At the molecular level, the increased expression of vascular adhesion molecules such as VCAM promote the  
15   adhesion of sickled red blood cells containing abnormal hemoglobin. It follows that decreasing cytokine expression on endothelial cells, promoting endothelial function and attenuating red cell sickling, are key therapeutic objectives. However, currently used therapies have been  
20   generally unsuccessful.

      In this novel method for loading red blood cells with intracellular NO-donor S-nitrosothiols, the effect is to increase oxygen affinity -- which in and of itself should attenuate red blood cell sickling -- and to endow the red  
25   blood cell with vasodilator and antiplatelet activity, which should reverse the vasoocclusive crisis. Moreover, nitric oxide should attenuate the expression of adhesion molecules on endothelial cell surfaces, thus restoring endothelial function.

30       Herein is described a novel therapeutic approach to the treatment of sickle cell disease which involves loading of red blood cells with S-nitrosothiols or other nitrosating agents. Two examples of therapeutic approaches

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are given. In the first, the patient's own red blood cells are S-nitrosated extracorporeally (yielding "loaded" red blood cells) and then given to the patient. The second approach is to directly administer to a patient an agent  
5 such as S-nitrosocysteine, which is permeable to red blood cells.

For some diseases or disorders, the administration of NO-loaded red blood cells is especially desirable. Upon a change from the oxygenated to the deoxygenated state, or  
10 upon a change in the oxidation state of the heme Fe from the reduced state (FeII) to the oxidized (FeIII) state, NO is released from the thiol groups of hemoglobin, and is rapidly transferred to glutathione to form S-nitrosoglutathione. Red blood cells are known to have a  
15 high concentration of glutathione. S-nitrosoglutathione efficiently delivers NO to tissues.

In another aspect, the invention is a method for the treatment of infection by administering to an infected mammal an agent which causes S-nitrosation of thiol groups  
20 within the cells which are the target of such agent. For example, an S-nitrosothiol to which lymphocytes are highly permeable can be administered to a patient infected with HIV. Such treatment for HIV can also be used extracorporeally, to blood isolated from the patient. In  
25 another application, the infection is bacterial, and the S-nitrosothiol to be used as an anti-bacterial agent is one to which the target bacterial cells are highly permeable, as compared to the permeability properties of the host cells. (See, for example De Groote, M.A., et al., Proc.  
30 Natl. Acad. Sci. USA 92:6399-6403 (1995).) Alternatively, nitrosothiols can be used to treat *Plasmodium falciparum* within red blood cells.

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Another embodiment of the invention is a method for specifically modifying a protein containing one or more metal atoms so that the protein becomes S-nitrosylated at one or more thiol groups without modifying the metal, as by  
5 changing the oxidation state or causing the metal atoms to bind NO. This can be accomplished by the use of a reagent which possesses NO<sup>+</sup> character, such as a nitrosothiol (See, for instance, Example 4A.), which reacts specifically with thiol groups of a protein in which metal is bound.

10 An S-nitrosation method has been devised which does not affect the heme of hemoglobin. SNO-Hb (SNO-Hb(FeII)O<sub>2</sub>) can be synthesized from Hb(FeII)O<sub>2</sub> with up to 2 SNO groups per tetramer without, or with only minimal, oxidation of the heme Fe from FeII to FeIII. Preferably, the proportion  
15 of metHb in such a SNO-Hb(FeII)O<sub>2</sub> composition is less than about 10%, more preferably, less than about 5%, and still more preferably, less than about 2%. In contrast, when Hb(FeII)O<sub>2</sub> is incubated with excess nitric oxide or nitrite, methemoglobin (HbFe[III]) forms rapidly (Example  
20 1B) and to a significant extent. When Hb[FeII] is incubated with nitric oxide, NO binds rapidly to the heme, forming Hb(FeII)NO to a significant extent (Example 1A).

Although rates of formation of SNO-Hb(FeII)O<sub>2</sub> from Hb(FeII)O<sub>2</sub> are more rapid (see Example 2A), the  
25 corresponding SNO-deoxyHb form can also be made by incubation of S-nitrosogluthathione or S-nitrosocysteine, for example, with Hb(FeII), yielding SNO-Hb(FeII), as in Example 1C. Preferably, the proportion of metHb found in such a SNO-Hb(FeII) composition is less than about 10%,  
30 more preferably, less than about 5%, and still more preferably, less than about 2%.

The effects of the various forms of Hb on vasodilation -- constriction, dilation or a neutral effect -- depend on

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three factors: whether 1) the Fe of the heme is oxidized, 2)  $O_2$  is bound at the heme (that is, the oxygenation state, dictated by the conformation of the protein as R state or T state), and 3) thiol is present in sufficient concentration  
5 to facilitate the transfer of  $NO^+$ .

The importance of the first factor is shown in Figure 4A.  $Hb(FeII)O_2$  and  $SNO-Hb[FeII]O_2$  act as vasoconstrictors, but  $SNO-Hb[FeIII]$  (metHb form, where FeII has been oxidized to FeIII) acts as a vasodilator. Figure 4A shows that  $SNO-$   
10  $Hb[FeII]O_2$  with oxygen bound at the heme, and with a ratio of  $SNO/Hb=2$ , acts as a powerful vasoconstrictor.

$SNO-Hb(FeII)$  is also a vasodilator. Figure 2B illustrates the second factor in demonstrating that rates of RSNO decomposition and transfer are much faster for  $SNO-$   
15  $Hb$  in the deoxy state than for  $SNO-Hb$  in the oxy state.

It can be seen how the  $NO^+$ -donating properties of  $SNO-Hb$  depend on oxygen concentrations.  $SNO-Hb$  releases oxygen at sites of low oxygen concentration or under oxidizing conditions.  $SNO-Hb$  releases its  $NO$  group(s) to cause  
20 vasodilation either due to 1) oxidation of the heme Fe to FeIII or 2) loss of the  $O_2$  from the heme by deoxygenation. It is shown in Figure 2B that  $NO$  is transferred off  $SNO-Hb$  best in the deoxy state. In ischemia,  $SNO-Hb$  deoxygenates, rapidly followed by the loss of  $NO$ . It can be seen from  
25 the data that  $SNO-metHb$  having a ratio of 1  $SNO/SNO-metHb$  is a more powerful vasodilator than  $SNO-oxyHb$  having a ratio of 2  $SNO/SNO-oxyHb$ . It should be noted that S-nitrosation of Hb induces the R state (oxy conformation). Thus, it follows that 1  $SNO-oxyHb$  molecule having a ratio  
30 of 1  $SNO/SNO-oxyHb$  is less potent than 10 molecules of  $SNO-oxyHb$  having a ratio of 0.1  $SNO/SNO-oxyHb$ .

The third factor is illustrated by the results shown in Figure 4B. These results demonstrate potentiation by

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thiol of the vasodilator effect of SNO-Hb(FeII)O<sub>2</sub> and SNO-Hb(FeIII). Transfer of NO<sup>+</sup> from SNO-Hb to low molecular weight nitrosothiols is more efficient when Hb is in the deoxy state compared to the oxy state (Figure 2B) or in the met state compared to the oxy state (Figure 4C).

NO is released or transferred as NO<sup>+</sup> (nitrosyl cation) from SNO-Hb. The SNO groups of SNO-Hb have NO<sup>+</sup> character. Transfer of NO<sup>+</sup> from SNO-Hb occurs most efficiently to low molecular weight thiols, such as glutathione, and is most efficient when the heme is oxidized (SNO-metHb) or the SNO-Hb is in the deoxy state.

A nitrosating agent, especially one which can readily enter cells, especially red blood cells, can be used to treat a mammal with a disease or medical disorder which can be alleviated by increased biologically active NO in the affected tissues, such as sepsis, shock, angina, stroke, reperfusion injury, acute lung injury, sickle cell anemia, infection of red blood cells, and organ transplantation. One embodiment of the invention resulting from the findings herein is a method of therapy that enhances the transfer of NO<sup>+</sup> from SNO-Hb to low molecular weight thiols, thereby delivering NO biological activity to tissues, by the coadministration of low molecular weight thiols, along with a form of SNO-Hb, to a mammal in need of the physiological effects of NO, such as one suffering from the above medical conditions. To further increase the effect of NO release it is preferred that the SNO- forms of metHb or deoxyHb (or an equivalent conformation or spin state) be administered with the thiol (See Figure 2B, for example.) A mixture of SNO-metHb and SNO-oxyHb, and possibly also thiol, or more specifically, an S-nitrosothiol, can also be used. The composition and proportion of these components depends on the disease state. For example, in sickle cell anemia, to

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achieve both enhanced O<sub>2</sub> delivery and NO delivery, a composition comprising SNO-oxyHb can be administered. Where no further delivery of O<sub>2</sub> is desirable, as in acute respiratory distress syndrome, for example, the SNO- forms of methHb and deoxyHb are especially preferred. Alternatively, the ratios of SNO/Hb can be regulated to control O<sub>2</sub> release.

A further invention arising out of the discoveries presented herein is a method for preserving a living organ ex vivo, for example for transplantation, comprising perfusing the organ with a composition comprising nitrosated hemoglobin and low molecular weight thiol or NO donating agent, wherein SNO-Hb(FeII)O<sub>2</sub> is a preferred nitrosated hemoglobin.

The vessel ring bioassay data of Figure 4A agree well with the in vivo data of Figure 5. The results of the experiments described in Example 5 confirm that Hb(FeII)O<sub>2</sub> (oxyHb) causes an increase in blood pressure in vivo, as it did also in vitro. SNO-Hb(FeIII) (SNO-metHb) causes a decrease in blood pressure in vivo as well as in vitro. SNO-Hb(FeII)O<sub>2</sub> (SNO-oxyHb) has a negligible effect on blood pressure in vivo in contrast to the increase in tension seen in the corresponding vessel ring bioassay. For SNO-oxyHb the in vivo effect is neutral. This is explained by the constrictive effect caused by NO becoming bound to the heme being compensated by the release of NO upon deoxygenation. Therefore, SNO-oxyHb can deliver O<sub>2</sub> with minimal effect on blood pressure.

With knowledge of the results herein it is possible to synthesize Hb proteins with predicted NO-releasing properties, which will constrict, dilate, or have no effect on blood vessels. An additional option is the choice between making oxygenated or deoxygenated forms to

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administer for medical conditions in which O<sub>2</sub> delivery is desirable, or undesirable, respectively.

It is possible to produce a variety of modified Hbs having specific desired properties of O<sub>2</sub> and NO delivery.

5 For example, Hb in the R state or R-structure (oxyHb) can be converted to the T state or T-structure (deoxyHb) by a number of known methods. This can be done, for example, by reaction of Hb with inositol hexaphosphate. It is also known to those skilled in the art that Hb in the R state  
10 can be made, for example, by treating Hb with carboxypeptidase. Similarly, it is known that metHb can be synthesized using ferricyanide or nitrite.

Producing Hb molecules which are locked in the T state allows the synthesis of RSNO-Hb which remains in a form  
15 that is a biologically active donor of NO, rather than a carrier of NO. Hb which is locked in the R state can be used as a substrate for the synthesis of RSNO-Hb which carries a maximum amount of NO per molecule.

Another embodiment of the invention is a blood  
20 substitute comprising one or more forms of Hb which have been specifically S-nitrosated to some extent at one or more thiol groups of the Hb, in order to regulate O<sub>2</sub> release and NO release. Conditions to be treated include those in which NO or O<sub>2</sub> delivery is desired, those in which  
25 NO or O<sub>2</sub> utilization is desired, or those in which NO or O<sub>2</sub> is in excess. For example, in a medical condition which is characterized by the presence of an excess of oxygen free radicals and excess NO·, both the heme of SNO-Hb and NO released by SNO-Hb serve to trap oxygen free radicals. The  
30 heme Fe is oxidized in the process of scavenging oxygen free radicals and NO·, and NO is released from the oxidized Hb by donation to a thiol, in the form of RSNO<sup>+</sup>, which is not toxic. Inflammation and reperfusion injury, for



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example, are characterized by excess NO production and an excess of oxygen free radicals. Forms of Hb scavenge oxygen radicals and free NO, converting NO to forms that are not toxic.

5       A further embodiment of the invention is a method of therapy for a condition that would benefit from the delivery of NO in a biologically active form or O<sub>2</sub> or both, based on the administration of a blood substitute comprising a form of nitrosated Hb, such as S-

10   nitrosohemoglobin, either alone or in combination with a low molecular weight thiol, for example. For example, SNO-Hb is useful to treat myocardial infarction. SNO-Hb has the effect of donating NO, keeping blood vessels open. SNO-Hb deoxygenates at low oxygen tension, delivering

15   oxygen and releasing NO at the same site, thereby causing vasodilation. (See Example 7 and Figures 6A-6F.) These effects can be augmented by also administering thiol, either simultaneously with SNO-Hb, or before or after. For the purpose of treating myocardial infarction, for example,

20   a high concentration or dose of SNO-Hb that has a low ratio of SNO/SNO-Hb is appropriate. Alternatively, SNO-metHb can be used for this purpose. A further application of these principals is a method for increasing cerebral blood flow in a mammal comprising administering to the mammal a

25   composition comprising S-nitrosohemoglobin, as illustrated in Figures 23A-23I.

      In another aspect, the invention is a method of enhancing NO-donor therapy by coadministering a composition comprising SNO-Hb or other forms of nitrosated Hb together

30   with a nitroso-vasodilator (nitroglycerin, for example) which would be otherwise consumed by the conversion of oxyHb to metHb in Hb which has not been S-nitrosated. A composition comprising a low molecular weight thiol can

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have the effect of producing a vasorelaxant response in a mammal (see Example 22 and Figure 20D). Any of the forms of isolated Hb described herein can be used in the manufacture of a medicament for the treatment of medical conditions characterized by abnormalities of nitric oxide and/or oxygen metabolism, as appropriate from the effects of the particular form or forms of Hb included in the medicament.

Platelet activation is manifested by a number of events and reactions which occur in response to adhesion of platelets to a nonplatelet surface such as subendothelium. Binding of agonists such as thrombin, epinephrine, or collagen sets in motion a chain of events which hydrolyzes membrane phospholipids, inhibits adenylate cyclase, mobilizes intracellular calcium, and phosphorylates critical intracellular proteins. Following activation, platelets secrete their granule contents into plasma, which then allow the linking of adjacent platelets into a hemostatic plug. (See pages 348-351 in *Harrison's Principles of Internal Medicine*, 12th edition, eds. J.D. Wilson et al., McGraw-Hill, Inc., New York, 1991).

A thrombus is a pathological clot of blood formed within the circulatory system. It can remain attached to its place of origin or become dislodged and move to a new site within the circulatory system. Thromboembolism occurs when a dislodged thrombus or part of a thrombus partially or completely occludes a blood vessel and prevents oxygen transport to the affected tissues, ultimately resulting in tissue necrosis.

Sites where damage has occurred to the vascular surface are especially susceptible to the adherence of platelets and the formation of thrombi. These sites include those on the interior surface of a blood vessel in

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which damage to the endothelium, narrowing or stenosis of the vessel, or atherosclerotic plaque accumulation has occurred.

NO is one of several endothelium-derived  
5 thromboregulators, which are defined as physiological substances that modulate the early phases of thrombus formation. In particular, NO reduces platelet adhesion, activation and recruitment on the endothelial cell surface, and achieves this, it is thought, by activating platelet  
10 guanylate cyclase, thereby increasing platelet intracellular CGMP (Stamler, J.S. et al, *Circ. Res.* 65:789-795 (1989)), and decreasing intraplatelet  $Ca^{2+}$  levels. NO and the prostacyclin prostaglandin (PG)  $I_2$  act synergistically to inhibit and actively mediate platelet  
15 disaggregation from the collagen fibers of the subendothelial matrix. Unlike prostacyclin, NO also inhibits platelet adhesion. Furthermore, platelets synthesize NO, and the L-arginine-NO pathway acts as an intrinsic negative feedback mechanism to regulate platelet  
20 reactivity. NO is involved in leukocyte interactions with the vessel wall and can inhibit neutrophil aggregation. (See review article, Davies, M.G. et al., *British Journal of Surgery* 82:1598-1610, 1995.)

NO is antiathrogenic in a number of ways. (See, for  
25 example, Candipan, R.C. et al., *Arterioscler. Thromb. Vasc. Biol.* 16:44-50, 1996.) NO inhibits smooth muscle proliferation and attenuates LDL (low density lipoprotein) oxidation and other oxidant-related processes.

Hemoglobin may promote atherosclerosis as well as  
30 thrombosis as a consequence of its NO-scavenging property. This limitation of hemoglobin derives from its high affinity for nitric oxide. In vitro, NO is a potent inhibitor of platelet aggregation and adhesion to collagen

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- fibrils, the endothelial cell matrix and monolayers (Radomski, M.W. et al., *Br. J. Pharmacol.* 92:181-187 (1987); Radomski, M.W. et al., *Lancet* 2:1057-1058 (1987); Radomski M.W. et al., *Biochem. Biophys. Res. Commun.* 148:1482-1489 (1987)). NO elevates CGMP levels in platelets, thereby decreasing the number of platelet-bound fibrinogen molecules and inhibiting intracellular  $Ca^{++}$  flux and platelet secretion (Mellion, B.T. et al., *Blood* 57:946-955 (1981); Mendelson, M.E. et al., *J. Biol. Chem.* 165:19028-19034 (1990); Lieberman, E. et al., *Circ. Res.* 68:1722-1728 (1991)). Scavenging of nitric oxide by Hb prevents the molecule from inhibiting platelets. This explanation has been given support by *in vivo* studies (Krejcy, K. et al., *Arterioscler. Thromb. Vasc. Biol.* 15:2063-2067 (1995)).

The results shown in Figures 7A-7C (see Example 9) show that nitrosated hemoglobins, including SNO-Hb, can be used in a therapeutically effective amount, in the treatment of acute blood clotting events that occur as a result of increased platelet deposition, activation and thrombus formation or consumption of platelets and coagulation proteins. Such complications are known to those of skill in the art, and include, but are not limited to myocardial infarction, pulmonary thromboembolism, cerebral thromboembolism, thrombophlebitis, sepsis and unstable angina, and any additional complication which occurs either directly or indirectly as a result of the foregoing disorders.

SNO-Hb and other nitrosated hemoglobins can also be used prophylactically, for example, to prevent the incidence of thrombi in patients at risk for recurrent thrombosis, such as those patients with a personal history or family history of thrombosis, with atherosclerotic

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vascular disease, with chronic congestive heart failure, with malignancy, or patients who are pregnant or who are immobilized following surgery.

NO is known to activate soluble guanylate cyclase, which produces cGMP. cGMP mediates inhibition of platelet aggregation. Results in Example 10 demonstrate that this inhibition of platelet aggregation may be mediated not by cGMP alone, but by some other mechanism as well.

Certain compounds or conditions are known to cause a shift in the allosteric equilibrium transition of Hb towards either of the two alternative quaternary structures of the tetramer, the T- or R-structures. (See, for example, pages 7-28 in Perutz, M., *Mechanisms of Cooperativity and Allosteric Regulation in Proteins*, Cambridge University Press, Cambridge, U.K., 1990.) These are, for instance, the heterotropic ligands  $H^+$ ,  $CO_2$ , 2,3-diphosphoglycerate (2,3-DPG) and  $Cl^-$ , the concentrations of which modulate oxygen affinity. The heterotropic ligands lower the oxygen affinity by forming additional hydrogen bonds that specifically stabilize and constrain the T-structure. Other compounds affecting the allosteric equilibrium include inositol hexaphosphate (IHP) and the fibric acid derivatives such as bezafibrate and clofibrate. The fibric acid derivatives, antilipidemic drugs, have been found to combine with deoxy-, but not with oxyhemoglobin. They stabilize the T-structure by combining with sites in the central cavity that are different from the DPG binding sites. Other allosteric effectors have been synthesized which are related to bezafibrate. A ligand that stabilizes specifically the R-structure increases the oxygen affinity, and a ligand that stabilizes the T-structure does the reverse. Other ligands can affect the spin state of the heme. For example, in deoxyhemoglobin and in methemoglobin

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the Fe is high-spin ferrous ( $S=2$ ) and 5-coordinated; in oxyhemoglobin and in cyan-metHb the Fe is low-spin ferrous ( $S=0$ ) and 6-coordinated; when  $H_2O$  is the sixth ligand, methemoglobin is also high-spin. The inhibition of platelet aggregation by S-nitroso-methemoglobin seen in Figure 7C is consistent with enhanced potency in the high spin conformation. Substances which control the allosteric equilibrium or spin state of hemoglobin can be administered in a pharmaceutical composition to a human or other mammal, in a therapeutically effective amount, to promote the formation of, or to stabilize, a particular allosteric structure and/or spin state of hemoglobin, thereby regulating platelet activation, e.g., by converting hemoglobin from R-structure to T-structure.

The dosage of Hb required to deliver NO for the purpose of platelet inhibition can be titrated to provide effective amounts of NO without causing drastic changes in blood pressure. If the goal of the therapy is to deliver oxygen, the Hb can be administered in a unit of blood to avoid a drop in blood pressure. If the goal is to alleviate shock, very little Hb can be administered compared to the amount to be given for myocardial infarction. For shock, the more important goal is to deliver NO rather than to deliver oxygen. For this objective, it can be preferable to use continuous infusion or several infusions per day. Example 12 (see Figure 10) shows that the effects of  $SNO-Hb(FeII)O_2$  on blood flow in rat brain last over 20 minutes; in other experiments an effect has been seen for up to an hour. There is a correlation between blood pressure effects and platelet inhibition effects, but platelet inhibition occurs at a lower NO concentration than that which is required to produce blood pressure effects, and generally lasts longer.

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Example 11 shows that S-nitrosothiols can be used to add NO groups not only on the thiol groups of cysteine residues in hemoglobin, but also on other reactive sites of the hemoglobin molecule. The products of the nitrosation reactions in Example 11 were hemoglobin molecules with more than 2 NO groups per Hb tetramer. The exact sites of the addition of NO have not been confirmed, but it is expected that NO addition occurs at thiol groups and various other nucleophilic sites within Hb, including metals. Reactive sites, after the thiol groups, are tyrosine residues and amines, and other nucleophilic centers.

Nitrosation reactions on other proteins have been investigated previously (Simon, D.I. et al., *Proc. Natl. Acad. Sci. USA* 93:4736-4741 (1996)). Methods of modifying proteins to produce nitrosoproteins are known in the art, and include, for example, exposing the protein to  $\text{NaNO}_2$  in 0.5 M HCl (acidified  $\text{NO}_2^-$ ) for 15 minutes at 37°C. An alternative method is to place a helium-deoxygenated solution of protein in 100 mM sodium phosphate, pH 7.4, inside dialysis tubing and expose the protein to NO gas bubbled into the dialysate for 15 minutes. (Stamler, J.S. et al., *Proc. Natl. Acad. Sci. USA* 89:444-448 (1992); see also Williams, D.L.H. *Nitrosation*, Cambridge University Press, New York (1988), which gives further methods of nitrosation).

By these methods, multiple NO-related modifications ("NO groups" or "NO biological equivalents" resulting from nitrosations, nitrosylations or nitrations) can be made on Hb at nucleophilic sites, which can include thiols, nucleophilic oxygen atoms as found in alcohols, nucleophilic nitrogen atoms as found in amines, or the heme iron. Agents which can be contacted with hemoglobin to facilitate nitrosations, nitrosylations or nitrations of Hb

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can be thought of as "NO or NO<sup>+</sup> donating agents." The products of such modifications may have such groups, for example, as -SNO, -SNO<sub>2</sub>, -ONO, ONO<sub>2</sub>, -CNO, -CNO<sub>2</sub>, -NNO, -NNO<sub>2</sub>, -FeNO, -CuNO, -SCuNO, SFeNO and the different ionized forms and oxidation variants thereof. (See, regarding oxidation of hemoglobin by Cu<sup>++</sup>, Winterbourne, C., *Biochemistry J.* 165:141-148 (1977)). The covalent attachment of the NO group to sulfhydryl residues in proteins is defined as S-nitrosation; the covalent attachment of the NO group to a metal, such as Fe, can be called nitrosylation, yielding a metal-nitrosyl complex. General NO attachment to nucleophilic centers is referred to herein as nitrosation. Thus, the term nitrosated hemoglobin as used herein includes SNO-Hb and Hb(FeII)NO as well as other forms of hemoglobin nitrosated at other sites in addition to thiols and metals. In addition, Hb can be nitrated. Compositions comprising Hbs which have been nitrosated and/or nitrated by a nitric oxide donating compound at multiple different types of nucleophilic sites (termed polynitrosated, that is, having NO equivalents added to other nucleophilic sites as well as to thiols; or polynitrated, respectively) will permit transnitrosation reactions and the release of NO and its biological equivalents in the circulatory system at different rates and engendering different potencies. Polynitrosated or polynitrated hemoglobins can be reacted with a reagent which selectively reduces FeIII to FeII (for example, cyanoborohydride or methemoglobin reductase), if it is desired to reduce heme Fe that may have been oxidized.

These and other nitrosation and nitration reactions can cause oxidation of the heme Fe to some extent, under some conditions. However, some minor degree of oxidation is acceptable. The nitrosated Hb is still be useful as a



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therapeutic agent if oxidized to a minor extent. For applications where the NO-delivering function, rather than the O<sub>2</sub>-delivering function of nitrosated Hb, is more desirable, extensive oxidation of the heme Fe is acceptable.

If it is desired to avoid oxidation of the heme Fe, it is possible to remove the heme, perform the necessary chemical reactions upon the protein to nitrosate to the extent desired, and replace the heme into the modified hemoglobin product. (See, for removing and replacing the heme, Antonini, E. and Brunori, M., *Hemoglobin and Myoglobin in their Reactions with Ligands*, Elsevier, New York, 1971.)

In addition to the nitrosating under conditions that do not oxidize the heme, such as brief exposure to low molecular weight RSNOs, as illustrated in Examples 1 and 2, alternative methods can be used to produce nitrosated hemoglobin in which the heme Fe is not oxidized. For instance, it is possible to produce by recombinant methods  $\alpha$  and  $\beta$  globin chains, nitrosate them to the extent desired, then assemble the chains with heme to form a functional, nitrosated tetramer. (See, for example, European Patent Application EPO 700997, published March 13, 1996, "Production in bacteria and yeast of hemoglobin and analogues thereof.")

Another alternative method to nitrosate the  $\alpha$  and  $\beta$  globin chains without producing a form of methHb as the end product, is to nitrosate the intact Hb molecule to the extent desired, thereby allowing the heme Fe to be oxidized, then reduce the heme Fe from FeIII to FeII by treating the nitrosated Hb with either methemoglobin reductase or a cyanoborohydride such as sodium cyanoborohydride.

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It has been generally thought that nitric oxide as NO gas in solution reacts with hemoglobin (Hb) in two major ways: 1) with the deoxyHb to form a stable nitrosyl (FeII) heme adduct; and 2) with oxyHb to form nitrate and methHb -- a reaction that inactivates NO. These two reactions contributed to the idea that Hb is a scavenger of NO. In both of these reactions, NO biological activity is lost. The results described herein demonstrate that, in fact, neither reaction occurs under physiological conditions. Rather, the products of the NO/Hb reaction are dictated by the ratio of NO to Hb, and by the conformation of Hb -- R(oxy) vs. T(deoxy).

At low ratios of NO to deoxyHb (e.g., 1:100 or less), the Hb molecule is in T-structure. Under this condition, NO introduced as gas to a Hb solution binds to the  $\alpha$ -hemes, as has been seen by EPR. Upon introduction of oxygen, with conversion to the R state, NO is transferred to a thiol of cysteine to yield S-nitrosohemoglobin with close to 100% efficiency. At ratios of NO/Hb of 1:25-1:50, the efficiency of formation of SNO-Hb is ~35% (decreasing with increasing NO/Hb ratio). The reaction appears to involve migration of NO from  $\alpha$  heme to  $\beta$  heme and then to the  $\beta$  thiol. In going from the heme to a thiol, the heme or nitrosothiol needs to lose an electron by oxidation ( $\text{NO} \rightarrow \text{NO}^+$  or  $\text{RSNO} \cdot \rightarrow \text{RSNO}$ ). Oxygen serves as an electron acceptor in the system, driving the reaction thermodynamically, as well as causing a conformational change by its binding at the heme, which exposes the thiol groups. At higher ratios of NO to Hb (1:20-1:2), with the protein still in T-structure, the protein liberates  $\text{NO}^-$  from the  $\beta$  hemes with production of methHb. This occurs in the absence of  $\text{O}_2$  and provides another indication that the NO bound to  $\beta$ -hemes is unstable. Once  $\text{O}_2$  is introduced, S-

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nitrosothiol (SNO) forms, but the relative yield is very low because of loss to  $\text{NO}^-$ . The yield of SNO-Hb approaches zero at NO/Hb ratios of 1:2, upon introduction of oxygen.

At the higher ratios of NO to Hb (i.e.,  $>0.75$ -1), NO  
5 itself maintains the R-structure. Under this condition, the NO is more stable because of an unusual constraint on the molecule. Specifically, loss of NO from the  $\beta$  hemes promotes the T-structure, whereas formation of SNO-Hb selects for the R-structure. This is not a favored  
10 reaction. The consequence is that small amounts of S-nitrosohemoglobin are formed, but the yields are low (~5%). This does not exclude the possibility that the molecule has therapeutic value.

The reaction of NO with oxyHb is also dependent on the  
15 ratios of NO to oxyHb. Under conditions of relatively high (non-physiological) ratios of NO to Hb, (NO/oxyHb  $>1:20$ ), NO appears to destabilize the hydrogen bond between the  $\text{O}_2$  and the proximal histidine (by competing for it) yielding some metHb. By changing the ionic composition of the  
20 solvent buffer (e.g., borate 0.2 M, pH 7.4), formation of metHb can be significantly reduced even with excess NO (NO/Hb = 3:1). On the other hand, metHb formation is facilitated in acetate buffer at pH 7.4; when the hydrogen bond between  $\text{O}_2$  and the proximal histidine is broken, the  
25  $\text{O}_2$  seems to gain superoxide-like character. NO then reacts rapidly to form metHb and nitrate. Efficient metHb formation actually requires an excess of NO/oxyHb. In contrast, at lower ratios of NO/Hb ( $<1:20$ ), it reacts with the small residual fraction ( $<1\%$ ) of deoxyHb, in turn  
30 producing S-nitroso-hemoglobin extremely efficiently. As the concentration of NO is increased, there is some reaction with oxyHb, but the products are nitrite and nitrate, not nitrate alone. The conclusion is that NO can

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be incubated in reaction mixtures of oxyHb without inactivating the O<sub>2</sub> binding functionality by converting it to nitrate.

Nitrosylhemoglobin can be used in an animal or human  
5 as a therapeutic NO donor for the prevention or treatment of diseases or medical disorders which can be alleviated by delivery of NO or its biologically active form to tissues affected by the disease or medical disorder. Like SNO-Hb, nitrosylhemoglobin can be administered as a blood  
10 substitute, because nitrosylhemoglobin can be converted to SNO-Hb under physiological conditions. NO is released from the thiol either by deoxygenation or by conversion to metHb.

An illustration of nitric oxide reactions with  
15 hemoglobin in the respiratory cycle is presented in Figure 26. The upper panel in Figure 26 shows alternative reactions proposed for  $\beta$ -chain nitrosyl hemes in the T structure. (1) Transfer of NO to the  $\alpha$ -chain heme irons (this is likely to occur mainly in the microcirculation and  
20 venous system; (2) charge-transfer reaction at the heme iron to produce methemoglobin and nitroxyl anion (this is more likely to occur in the microcirculation and venous system when NO synthesis is high); and (3) NO group exchange with  $\beta$ -Cys93 mediated by the oxygen-driven  
25 allosteric transition to the R structure, forming SNO-Hb(FeII)O<sub>2</sub> (this seems to occur in the lung, but may also happen in oxygenated arterial blood). See Example 15, for instance. Squares, circle and diamond represent hemes in the T structure, R structure and met state, respectively.

30 The lower panel of Figure 26 shows a model of NO binding to hemes and thiols of Hb in the circulation. Partially nitrosylated venous blood enters the lungs in the deoxy or T structure (square) (presumably maintained by  $\alpha$ -

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chain binding of NO to hemes and CO<sub>2</sub> to amines). Specifically, NO can be detected in large veins as  $\alpha$ (5- and 6- coordinate) nitrosyl heme (Westenberger, U. et al., *Free Rad. Res. Commun.* 11:167-178 (1990); Hall, D.M. et al., *J. Appl. Physiol.* 77:548-553 (1994); Kosaka, H. et al., *Am. J. Physiol.* 266:1400-1405 (1994); and Kagan, V.E. et al., *Nature* 383:30-31 (1996)), some of which is found in the fraction of blood that is fully deoxygenated. In the lungs, partially nitrosylated (carbamino) Hb is exposed to more NO and O<sub>2</sub> tensions (pO<sub>2</sub>) that appear to couple the allosteric transition with NO group exchange from hemes to  $\beta$ -chain thiols. (For example, see Examples 24 and 25.) Oxygen serves both to position the  $\beta$  thiol close to the  $\beta$  heme (Riggs, A. and Wolbach, R.A., *J. Gen Physiol.* 39:585-605 (1956)) and thermodynamically to drive the redox mediated formation of SNO. Accordingly, blood entering the arterial circuit contains SNO-oxy Hb, that is, Hb in the R structure (circle) with NO attached to  $\beta$ -Cys93 and O<sub>2</sub> to the hemes. Low-molecular-mass SNOs present in the lung and arterial blood will further support SNO-Hb formation by transnitrosation of R-structure molecules (Example 1). Blood moving into resistance vessels that control blood pressure and blood flow to tissues is then exposed to low pO<sub>2</sub> which promotes the T structure in SNO-Hb and effects NO group release. Some NO will exchange with low-relative-mass thiols to dilate blood vessels and some will be autocaptured at the hemes ( $\beta \rightarrow \alpha$ ; just as some endothelial-derived NO is inevitably sequestered by the hemes; see Example 3). NO oxidation of heme irons (methHb formation) will also enhance the vasodilator function of SNO-Hb. For instance, see Example 4. As O<sub>2</sub> delivery is a function of blood flow, the R  $\rightarrow$  T transition in Hb (and perhaps methHb formation) is designed to maximize oxygen delivery. Hb can

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further bind NO at the heme irons as it progresses through the venous system; the more NO that binds, the greater the propensity to form methHb and hemoglobin X

[Hb( $\alpha$ FeIIINO)( $\beta$ FeIII)]. It is not known whether the

5 endogenous level of ~0.1% nitrosyl Hb, which should promote the T structure in Hb, is sufficient to enhance O<sub>2</sub> delivery, but higher levels found in endotoxic shock (Kosaka, H. et al., *Am. J. Physiol.* 266:1400-1405 (1994)) may do so.

10 Inhaled NO causes selective pulmonary vasodilation without influencing systemic responses. A previously-formed rationale behind its use is that scavenging by Hb prevents adverse systemic effects. It is illustrated in Examples 14-21 that NO can be used to produce S-  
15 nitrosohemoglobin, which is a potent vasodilator and antiplatelet agent. Inhaled NO can be used to raise levels of endogenous S-nitrosohemoglobin. Similarly, treatment of red blood cells (RBCs) with NO can be used to form SNO-RBCs, or "loaded" red blood cells.

20 Compared to SNO-deoxyHb, which is a good NO donor, but which would release its NO very quickly, or SNO-oxyHb, which would release its NO more slowly, but has a propensity to form methHb over time, nitrosyl-deoxyhemoglobin stored in a form such that final ratio of  
25 NO:heme is less than about 1:100 or greater than about 0.75, is stable. Formation of methHb is prevented at these NO:heme ratios. For this reason nitrosyl-deoxyhemoglobin stored with such NO:heme ratios in a physiologically compatible buffer can be administered to an animal or human  
30 as an NO donor. Erythrocytes comprising nitrosylhemoglobin can also be used as NO donors. Erythrocytes comprising nitrosylhemoglobin can be made in a process comprising

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incubating deoxygenated erythrocytes in a solution comprising NO.

A blood substitute or therapeutic which can be used as an NO donor, and which is free of the vasoconstrictor effects of underviatized Hbs, can be made by obtaining a solution of oxyHb (including solutions stored in the form of oxyHb) and adding NO as dissolved gas, yielding SNO-oxyHb. Buffer conditions and NO:Hb ratios can be optimized, as illustrated in Example 21 and Figure 19, to yield S-nitrosothiol without significant production of oxidized Hb (metHb). For example, NO added to oxyhemoglobin in 10 mM phosphate buffer, pH 7.4, at a ratio of less than 1:30 NO:Hb resulted in formation of SNO-oxyHb with minimal formation of metHb. This ratio can be increased by varying the buffer conditions, for example by the use of 10 mM phosphate, 200 mM borate at pH 7.4. The buffer anions as well as the buffer concentration should be chosen carefully. For instance, acetate and chloride have the opposite effect from borate, increasing the formation of metHb and nitrite at 200 mM, pH 7.4.

This can be explained by a competition between free NO and oxygen for a H-bond with the imidazole of the proximal His residue. If low concentrations of NO are used, in low ionic strength buffer, e.g., 10 mM phosphate, metHb does not readily form. If the H-bond is weakened by increasing the ionic strength of the buffer, NO reacts more readily with oxyHb, yielding more metHb. Buffers with a low  $pK_a$  relative to pH 7.4 tend to stabilize FeIII. Buffers having a  $pK_a$  at least about two pH units higher than the reaction condition are preferred.

A blood substitute can be made which acts as a donor of NO<sup>-</sup>. NO can be added to a solution of deoxyHb at a ratio of NO:Hb in the range of 1:100 to 1:2, with a ratio

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of NO/heme of approximately 1:10 being preferred. If the ratio of NO:heme is increased, to a NO:Hb of about 2 (at which Hb is still in the T (deoxy) state), in the absence of an electron acceptor/free radical scavenger, NO is released from the  $\beta$  heme as  $\text{NO}^-$ , with oxidation of the heme iron to form metHb. The product solution can be used as a blood substitute or a therapeutic NO donor.  $\text{NO}^-$  can protect from N-methyl-D-glutamate-mediated brain injury in stroke; this effect has not been found for NO.

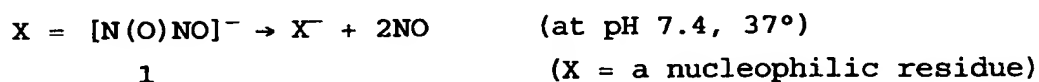
10 Nitrosylhemoglobin belongs to a broader class of nitrosyl-heme-containing donors of NO which can be administered to an animal or human for the delivery of NO to tissues. Nitrosyl-heme-containing donors of NO include, for example, the nitrosated ("nitrosated" as defined  
15 herein) hemoglobins nitrosylhemoglobin and SNO-nitrosylhemoglobin, nitrosyl-heme, and substituted forms of hemoglobin in which a different metal, (e.g.,  $\text{Co}^{++}$ ,  $\text{Mg}^{++}$ ,  $\text{Cu}^{++}$ ) is substituted for the heme iron, or nitrosyl-porphyrins are substituted for the heme.

20 Applicants teach physiologically significant results that provide a rationale for NO donors to be attached to Hb. Such derivatized Hbs can themselves serve as NO-donating therapeutics and can ameliorate the side effects of underivatized Hb administered as a blood substitute, for  
25 example. At one time, it had been thought that there would be no use for these compounds, because it was thought that NO released by the Hb would immediately be scavenged by the heme. It had been thought also that the released NO would oxidize Hb and limit oxygen delivery. The same rationale  
30 has previously limited the administration of NO donors, such as nitroglycerin and nitroprusside, because they had been thought to cause the formation of metHb.



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Preferably, NO-donors to be covalently attached to hemoglobin are relatively long-lived and have at least one functional group that can be used for the chemical attachment to hemoglobin. Examples of NO-donors include  
5 nitroprusside, nitroglycerin, nitrosothiols, and the diazeniumdiolate class of compounds (also called "NONOates") having structure 1.



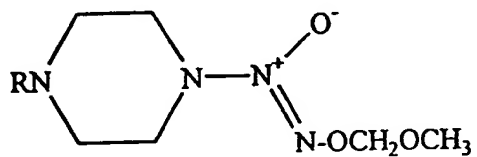
10 a diazeniumdiolate

A variety of these compounds have been synthesized that, in their anionic form, release NO without activation at physiological pH (Keefer, L.K. et al., *Am. Chem. Soc. Symposium Ser.* 553:136-146 (1994); Hanson, S.R. et al.,  
15 *Adv. Pharmacol.* 34:383-398 (1995)). Systemic administration can result in system-wide effects, according to equation 1. However, attachment to hemoglobin can be used to produce tissue-selective delivery of NO and oxygen. For instance, covalently esterified NO-donors can be  
20 activated predominantly in the liver. Different NO donors can be chosen to be linked to hemoglobin for different controlled release rates of NO from Hb.

Compound 4 (below), for example, is a diazeniumdiolate with a half-life for NO release, at 37°C and pH 7.4, of  
25 approximately two weeks. It can be converted to its nucleophilic N-4 mercaptoethyl derivative, compound 5. Hemoglobin can be activated toward coupling reactions by reacting it with  $\gamma$ -maleimidobutyric acid N-hydroxysuccinimide ester. Compound 5 can then be  
30 covalently attached to the activated hemoglobin through its maleimide functionality. The adduct, 6, can generate NO

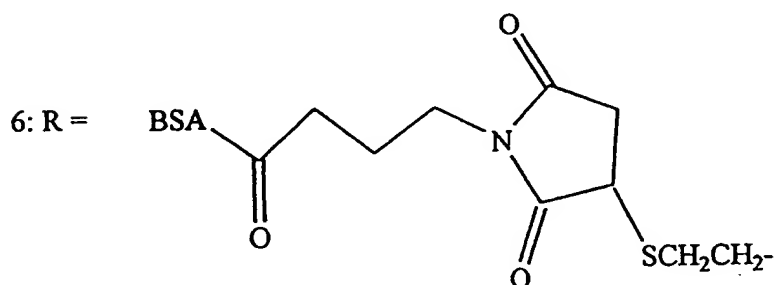
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steadily over several days in pH 7.4 phosphate buffer at 37°C. This property can alleviate side effects of underivatized blood substitutes, for example.



4: R = H-

5: R = HSCH<sub>2</sub>CH<sub>2</sub>-



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Nitric oxide synthase (NOS) working in conjunction with Hb can reload NO onto the hemes, thus a composition comprising NOS and Hb, or NOS conjugated to Hb can facilitate delivery of NO to the tissues. NOS of neurons is preferable for this composition because the neuronal NOS responds to oxygen tension. At low oxygen tension, the neuronal NOS produces more NO; at high oxygen tension, NOS produces less NO. This form of NOS will efficiently reload NO onto the heme when Hb is deoxygenated. NOS-Hb conjugates can be used when a blood substitute is indicated, and especially when an ischemic injury or condition is present.

Biologically compatible electron acceptors, are well known in the art and include, but are not limited to, superoxide dismutase and the oxidized forms of nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ), nicotinamide adenine dinucleotide phosphate ( $\text{NADP}^+$ ), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), ascorbate, dehydroascorbate and nitroxide spin traps. One or more electron acceptors can be conjugated to Hb molecules, and can facilitate the conversion of the nitrosyl-Hb-electron acceptor form to the SNO-Hb-electron acceptor form by accepting the electron lost by NO in its transfer, in the form of  $\text{NO}^+$  or as  $\text{RSNO}^\cdot$ , to a  $\beta 93\text{Cys}$  thiol group.

Nitroxides are one such class of electron acceptors which also act as free radical scavengers. Nitroxides are stable free radicals that have been shown to have antioxidant catalytic activities which mimic those of superoxide dismutase (SOD), and which when existing in vivo, can interact with other substances to perform catalase-mimic activity. Nitroxides have been covalently attached to hemoglobin. See Hsia, J-C., U.S. Serial No.

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5,591,710, the contents of which are incorporated by reference in their entirety. See also Liebmann, J. et al., *Life Sci.* 54:503-509 (1994), describing nitroxide-conjugated bovine serum albumin and differential nitroxide concentrations among the different organs of mice tested with the conjugate.

Methods for chemically attaching superoxide dismutase (SOD) to Hb are known in the art. For example, see Quebec, E.A. and T.M. Chang, *Artif. Cells Blood Substit. Immobil. Biotechnol.* 23:693-705 (1995) and D'Agnillo, F. and Chang, T.M., *Biomater. Artif. Cells Immobilization Biotechnol.* 21:609-621 (1993). SOD attached to nitrosylhemoglobin can drive the reaction in which NO is transferred from the heme to thiol, by serving as an electron acceptor.

Like NO, CO is known to have vasodilator effects. (See Zakhary, R. et al., *Proc. Natl. Acad. Sci USA* 93:795-798 (1996).) A solution of deoxyhemoglobin can be derivatized with CO by exposing it to purified CO gas in solution, until the desired extent of CO-bound Hb is reached. CO-derivatized Hb can be administered as a blood substitute or co-administered with other heme-based blood substitutes to alleviate the effects (e.g., hypertension, intestinal pain and immobility) of underivatized hemoglobin. Hemoglobins can be derivatized to the extent necessary to overcome constrictor effects, for example to a ratio of CO/Hb in the range of approximately 0.1% to 10%.

Because the  $\alpha$  subunits lack thiol groups to serve as NO<sup>+</sup> acceptors from the heme, a blood substitute comprising  $\alpha$  chains, for example in the form of dimers or tetramers, can be made which has different properties from a blood substitute comprising  $\beta$  chains alone, or comprising a combination of  $\alpha$  and  $\beta$  chains. A blood substitute comprising  $\alpha$  chains of hemoglobin can be administered to an

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animal or to a human patient to alleviate a condition characterized by the effects caused by NO, for example, in hypotensive shock.

$\beta$  chains, unlike  $\alpha$  chains, serve as active donors of NO to the tissues, rather than traps for NO. A blood substitute comprising  $\beta$  chains, for example in the form of  $\beta$  dimers or tetramers, can be made. Such a blood substitute can be administered to a mammal to treat diseases or medical disorders wherein it is desired to deliver oxygen as well as NO or its biological equivalent to tissues affected by the disease, for example, in angina and other ischemic conditions.

Methods are known by which hemoglobin can be separated into its  $\alpha$  and  $\beta$  subunits and reconstituted. Separated, heme-free, alpha- and beta-globins have been prepared from the heme-containing alpha and beta subunits of hemoglobin. (Yip, Y.K. et al., *J. Biol. Chem.* 247:7237-7244 (1972)). Native human hemoglobin has been fully reconstituted from separated heme-free alpha and beta globin and from hemin. Preferably, heme is first added to the alpha-globin subunit. The heme-bound alpha globin is then complexed to the heme-free beta subunit. Finally, heme is added to the half-filled globin dimer, and tetrameric hemoglobin is obtained (Yip, Y.K. et al., *Proc. Natl. Acad. Sci. USA* 74:64-68 (1997)).

The human alpha and beta globin genes reside on chromosomes 16 and 11, respectively. Both genes have been cloned and sequenced, (Liebhaber, et al., *Proc. Natl. Acad. Sci. USA* 77:7054-7058 (1980) (alpha-globin genomic DNA); Marotta, et al., *J. Biol. Chem.* 252:5040-5053 (1977) (beta globin cDNA); Lawn, et al., *Cell* 21:647 (1980) (beta globin genomic DNA)).

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Recombinant methods are available for the production of separate  $\alpha$  and  $\beta$  subunits of hemoglobin. For instance, Nagai and Thorngerson, (*Nature* 309:810-812 (1984)) expressed in *E. coli* a hybrid protein consisting of the 31 amino  
5 terminal residues of the lambda cII protein, an Ile-Glu-Gly-Arg linker, and the complete human beta globin chain. They cleaved the hybrid immediately after the linker with blood coagulation factor Xa, thus liberating the beta-globin chain. Later, (Nagai, K. et al., *Proc. Natl. Acad.*  
10 *Sci. USA* 82:7252-7255 (1985)) took the recombinant DNA-derived human beta globin, naturally derived human alpha globin, and a source of heme and succeeded in producing active human hemoglobin.

An efficient bacterial expression system for human  
15 alpha globin was reported. (GB 8711614, filed May 16, 1987; see also WO 88/09179). This led to the production of wholly synthetic human hemoglobin by separate expression of the insoluble globin subunits in separate bacterial cell lines, and *in situ* refolding of the chains in the presence  
20 of oxidized heme cofactor to obtain tetameric hemoglobin. A synthetic human hemoglobin has been produced in yeast cells (EP 700997A1, filing date 10.05.1990).

The properties of hemoglobin have been altered by specifically chemically crosslinking the alpha chains  
25 between the Lys99 of alpha 1 and the Lys99 of alpha 2. (Walder, U.S. 4,600,531 and 4,598,064; Snyder, et al., *Proc. Natl. Acad. Sci USA* 84:84 7280-7284 (1987); Chatterjee, et al., *J. Biol. Chem.* 261:9927-9937 (1986)). This chemical crosslinking was accomplished by reacting bis  
30 (3,5-dibromosalicyl) fumarate with deoxyhemoglobin A in the presence of inositol hexaphosphate. The beta chains have also been chemically crosslinked. (Kavanaugh, M.P. et al., *Biochemistry* 27:1804-1808 (1988)). Such linking methods or

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other suitable methods can be adapted to methods of producing  $\alpha$  or  $\beta$  dimers or other multimers, or for the crosslinking of other polypeptides to the  $\alpha$  and  $\beta$  chains. (For further methods to derivatize proteins and to  
5 conjugate proteins, see Hermansoh, G.T., *Bioconjugate Techniques*, Academic Press, 1996.)

The term hemoglobin or Hb as used herein includes variant forms such as natural or artificial mutant forms differing by the addition, deletion and/or substitution of  
10 one or more contiguous or non-contiguous amino acid residues, or modified polypeptides in which one or more residues is modified, and mutants comprising one or more modified amino acid residues. Hb also includes chemically  
15 as fusion proteins, and truncated forms. It also includes Hbs of all animal species and variant forms thereof. The biological and/or chemical properties of these variant Hbs can be different from those of hemoglobins which are found naturally occurring in animals.

20 It will be appreciated that NO exists in biological systems not only as nitric oxide gas, but also in various redox forms and as biologically active adducts of nitric oxide such as S-nitrosothiols, which can include S-nitrosoproteins, S-nitroso-amino acids and other S-  
25 nitrosothiols (Stamler, J.S. *Cell* 78:931-936 (1994)).

A blood substitute can be a biologically compatible liquid which performs one or more functions of naturally occurring blood found in a mammal, such as oxygen carrying and/or delivery, NO carrying and/or delivery, and the  
30 scavenging of free radicals. A blood substitute can also comprise one or more components of such a liquid which, when infused into a mammal, perform one or more functions of naturally occurring blood. Examples of blood

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substitutes include compositions comprising one or more forms of hemoglobin. Such compositions can also include other biologically active components, such as a low molecular weight thiol, nitrosothiol or NO donating agents, to allow transnitrosation. Low molecular weight thiols (i.e., relative to proteins and other biological macromolecules) can include glutathione, cysteine, N-acetylcysteine, S-nitrosocysteinyglycine, S-nitrosocysteine, and S-nitrosohomocysteine.

10 The compounds and therapeutic compositions of this invention to be used in medical treatment are intended to be used in therapeutically effective amounts, in suitable compositions, which can be determined by one of skill in the art. Modes of administration are those known in the art which are most suitable to the affected site or system of the medical disorder. Intravenous infusion is a preferred mode of administration of various forms of hemoglobin to be used as a blood substitute. Suitable compositions can include buffers, carriers, stabilizers or inert ingredients known to those of skill in the art, along with biologically active component(s).

25 The term "therapeutically effective amount," for the purposes of the invention, refers to the amount of modified Hb and/or nitrosating agent which is effective to achieve its intended purpose. While individual needs vary, determination of optimal ranges for effective amounts of each compound to be administered is within the skill of one in the art. Research animals such as dogs, baboons or rats can be used to determine dosages. Generally, dosages required to provide effective amounts of the composition or preparation, and which can be adjusted by one of ordinary skill in the art, will vary, depending on the age, health, physical condition, sex, weight, extent of disease of the



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recipient, frequency of treatment and the nature and scope of the desired effect. Dosages for a particular patient can be determined by one of ordinary skill in the art using conventional considerations, (e.g. by means of an appropriate, conventional pharmacological protocol). For example, dose response experiments for determining an appropriate dose of a heme-based blood substitute can be performed to determine dosages necessary to produce a physiological concentration of approximately 1 nM to 100  $\mu$ M heme. Suitable pharmaceutical carriers or vehicles can be combined with active ingredients employed in a therapeutic composition, if necessary.

The present invention is further and more specifically illustrated in the following examples, which are not intended to be limiting in any way.

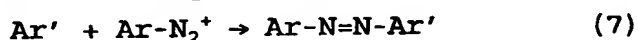
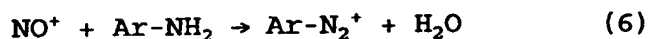
#### EXEMPLIFICATION

##### Materials and Methods for Assays

##### *Determination of R-S-NO Concentration (Standard Saville Method)*

The concentration of R-S-NO groups in a sample is based on the method reported in Saville, *Analyst* 83:670-672 (1958). The quantification of the NO group, displaced from the thiol by mercuric ion, forms the basis of this highly sensitive method. The detection limit is in the range of 0.1-0.5  $\mu$ M.

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As shown (equations 5-7), the reaction proceeds in two  
5 steps. First,  $\text{NO}^+$  is displaced from the RSNO by mercuric  
ion and reacts, under acidic conditions, with sulfanilamide  
( $\text{Ar-NH}_2$ ). In a second step, the diazonium salt (which is  
formed in amounts equivalent to the thionitrite) is then  
coupled with the aromatic amine, N-(1-naphthyl)-  
10 ethylenediamine ( $\text{Ar}'$ ), to form an intensely colored azo dye  
which can be measured at 540 nm ( $\epsilon \sim 50,000 \text{ M}^{-1} \text{ cm}^{-1}$ ). The  
same assay performed with the mercuric salt omitted allows  
for the simultaneous detection of nitrite. In principle,  
the second part of the Saville procedure is analogous to  
15 the classical Griess reaction for the detection of nitrite.

The procedure is as follows:

Solution A: sulfanilamide 1% dissolved in 0.5 M HCl.

Solution B: same solution as used in A to which 0.2%  $\text{HgCl}_2$

Solution C: 0.02% solution of N-(1-naphthyl)-

20 ethylenediamine dihydrochloride dissolved in 0.5 M HCl.

A given volume (50  $\mu\text{l}$ -1 ml) of the sample to be  
assayed is added to an equivalent volume of solution A and  
solution B. The two samples are set aside for 5 minutes to  
allow formation of the diazonium salt, after which an  
25 equivalent volume of solution C is added to each mixture.  
Color formation, indicative of the azo dye product, is  
usually complete by 5 minutes. The sample absorbance is  
then read spectrophotometrically at 540 nm. The RSNO is  
quantified as the difference in absorbance between solution  
30 B and A. (i.e. B - A). In the event that the background

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nitrite concentration is high (i.e. increased background in A), the accuracy of the measurement can be increased by the addition of an equivalent volume of 0.5% ammonium sulfamate in acid (45 mM) 5 minutes prior to the addition of  
5 sulfanilamide. The nitrous acid in solution reacts immediately with excess ammonium sulfamate to form nitrogen gas and sulfate.

Concentrations of thiol greater than 500  $\mu$ M in samples may interfere with the assay if nitrite is also present at  
10 micromolar concentration. Because nitrite will nitrosate indiscriminately under the acidic conditions employed, thiols will effectively compete for reaction with sulfanilamide (present at 50 mM in this assay) as their concentration approaches the millimolar range. This will  
15 lead to artifactual detection of RSNO. The problem can be avoided by (1) keeping the ratio of thiol to sulfanilamide < 0.01, (2) first alkylating thiols in the solution, or (3) adding free thiols to standards to correct for the potential artifact.

20 *Assay for S-Nitrosohemoglobin and Nitrosyl (FeII)-Hemoglobin*

A highly sensitive photolysis-chemiluminescence methodology was employed, which had been used for measuring RSNOs (S-nitrosothiols) in biological systems (Gaston, B., et al., *Proc. Natl. Acad. Sci. USA* 90:10957-10961 (1993);  
25 Stamler, J.S., et al., *Proc. Natl. Acad. Sci USA* 89:7674-7677 (1992)). The method involves photolytic liberation of NO from the thiol, which is then detected in a chemiluminescence spectrometer by reaction with ozone. The same principle of operation can be used to cleave (and  
30 measure) NO from nitrosyl-metal compounds (Antonini, E. and Brunori, M. *In Hemoglobin and Myoglobin in Their Reactions with Ligands*, American Elsevier Publishing Co., Inc., New

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York, pp. 29-31 (1971)). With adjustment of flow rates in the photolysis cell, complete photolysis of the NO ligand of Hb(FeII)NO is achieved. Standard curves derived from synthetic preparations of SNO-Hb, Hb(FeII)NO, and S-nitrosoglutathione were linear ( $R > 0.99$ ), virtually superimposable, and revealing of sensitivity limits of approximately 1 nM. Two analytical criteria were then found to reliably distinguish SNO-Hb from Hb(FeII)NO: 1) signals from SNO-Hb were eliminated by pretreatment of samples with 10-fold excess  $\text{HgCl}_2$ , while Hb(FeII)NO was resistant to mercury challenge; and 2) treatment of SNO-Hb with  $\text{HgCl}_2$  produced nitrite (by standard Griess reactions) in quantitative yields, whereas similar treatment of Hb(FeII)NO did not. UV/VIS spectroscopy confirmed that NO remained attached to heme in the presence of excess  $\text{HgCl}_2$ .

We linked a photolysis cell directly to the reaction chamber and detector portion (bypassing the pyrolyzer) of a chemiluminescence apparatus (model 543 thermal energy analyzer, Thermedix, Woburn MA). A sample (5 to 100  $\mu\text{l}$ ) is either introduced directly or introduced as a chromatographic effluent from an attached high-performance liquid or gas chromatography system into the photolysis cell (Nitrolite, Thermedix, Woburn MA). This cell consists of a borosilicate glass coil (3 m x 0.64 cm o.d. x 1 mm i.d., turned to a diameter of 6 cm and a width of 12 cm). The sample is introduced with a purge stream of helium (5 liters/min) and then irradiated with a 200-W mercury-vapor lamp (vertically mounted in the center of the photolysis coil on Teflon towers). The effluent from the photolysis coil is directed to a series of cold traps, where liquid and gaseous fractions less volatile than nitric oxide (such as nitrite and nitrate) are removed. Nitric oxide is then carried by the helium stream into the chemiluminescence

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spectrometer, in which free nitric oxide is detected by reaction with ozone. Signals are recorded on a digital integrator (model 3393A, Hewlett-Packard). Flow rates and illumination levels in the photolysis cell were designed to  
5 result in complete photolysis of the S-N bond of S-nitrosothiols, as confirmed by analysis of effluent from the cell according to the method of Saville (Saville, B., *Analyst* 83:670-672 (1958)).

To determine what fraction of the total nitric oxide  
10 detected in samples was derived from S-nitrosothiols, several control measurements were performed. Mercuric ion was used to displace nitric oxide selectively from the S-nitrosothiols (Saville, B., *Analyst* 83:670-672 (1958)). Comparison of measured nitric oxide concentrations from  
15 samples alternatively pretreated or not pretreated with HgCl<sub>2</sub> ensured that nitric oxide obtained by photolysis was derived specifically from S-nitrosothiols. Similarly, as an added measure of confirmation, we distinguished between S-nitrosothiols and free nitric oxide by comparing nitric  
20 oxide concentrations in samples alternatively exposed or not exposed to photolyzing illumination.

*Methods for Spectrophotometric Experiments and Nitrosylhemoglobin Formation, Examples 14-20*

Purified human HbA<sub>0</sub> was obtained from Apex Biosciences  
25 (Antonini, E. and Brunori, M. *In Hemoglobin and Myoglobin in Their Reactions with Ligands*, American Elsevier Publishing Co., Inc., New York (1971)). The spectrophotometer used was a Perkin Elmer UV/vis Spectrometer Lambda 2S. All measurements were made at 23°C  
30 in a sealed quartz cuvette to which all additions were made. Deoxygenation was achieved by argon passage through a Hb solution within a sealed quartz cuvette. The degree

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of deoxygenation can be measured by UV/vis spectrum. Nitrosylation of hemes is achieved by addition of purified NO gas to deoxyHb and the products quantitated by the extinction coefficient per Antonini and Brunori, *supra*.

5 Example 1: Interactions of NO and RSNO with Hb

It was observed that naturally occurring N-oxides, such as NO and RSNOs (Gaston, B., et al., *Proc. Natl. Acad. Sci. USA* 90:10957-10961 (1993); Scharfstein, J.S., et al., *J. Clin. Invest.*, 94:1432-1439 (1994); Clancy, R.M., et  
10 al., *Proc. Natl. Acad. Sci. USA* 91:3680-3684 (1994)), differed markedly in their reactions with Hb. NO bound very rapidly to deoxyHb (Hb[FeII]), forming relatively stable Hb[FeII]NO complexes (Figure 1A), and converted oxyHb (Hb[FeII]O<sub>2</sub>) to methemoglobin (Hb[FeIII]) and nitrate  
15 (Figure 1B), confirming previous reports (Olson, J.S., *Methods in Enzymol.* 76:631-651 (1981); Toothill, C., *Brit. J. Anaesthy.* 39:405-412 (1967)). In contrast, RSNOs were found to participate in transnitrosation reactions with sulfhydryl groups of Hb, forming S-nitrosohemoglobin (SNO-  
20 Hb), and did not react with the heme centers of either deoxyHb or Hb(FeII)O<sub>2</sub> (Figures 1C and 1D).

A. Interaction of NO with deoxyHb

Conversion of deoxyHb (Hb[FeII]) to Hb(FeII)NO is observed upon incubation of Hb(FeII) with increasing  
25 concentrations of nitric oxide. See Figure 1A. a. Deoxy Hb. b, c, d. Reaction mixtures of NO and Hb(FeII) in ratios of 1:1, 2:1 and 10:1, respectively. The reaction product Hb(FeII)NO formed essentially instantaneously on addition of NO (i.e. within instrument dead time).

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**B. Interaction of NO with oxyHb**

Conversion of oxyHb ( $\text{Hb}[\text{Fe}(\text{II})\text{O}_2]$ ) to metHb ( $\text{HbFe}(\text{III})$ ) is observed upon incubation of oxyHb with increasing concentrations of NO. See Figure 1B. a. oxy Hb. b, c, d. Reaction mixtures containing NO and oxyHb in ratios of 1:1, 2:1 and 10:1, respectively. Methemoglobin formation occurred instantaneously on addition of NO (i.e. within instrument dead time).

**C. Interaction of S-nitrosothiols with deoxyHb**

Conversion of  $\text{Hb}(\text{FeII})$  to SNO-Hb( $\text{FeII}$ ) is observed upon incubation of either GSNO (shown) or S-nitrosocysteine (CYSNO) with deoxy Hb. There is little (if any) interaction of RSNO with the heme functionalities of Hb. See Figure 1C. a. deoxyHb. b, c, d. Reaction mixtures of GSNO and  $\text{Hb}(\text{FeII})$  in ratios of 1:1, 2:1 and 10:1, respectively. Spectra were taken after 60 min of incubation in b, c, and 15 min in d. Further analysis of reaction products revealed the formation of moderate amounts of SNO-Hb in all cases. Yields of SNO-Hb (S-NO/Hb) in b, c, and d at 60 min were 2.5%, 5% and 18.5%, respectively. (See Figure 1D and Figure 2A.)

**D. Interaction of S-nitrosothiols with oxyHb**

Conversion of  $\text{Hb}(\text{FeII})\text{O}_2$  to SNO-Hb( $\text{FeII})\text{O}_2$  is observed upon incubation of either GSNO (shown) or CYSNO with oxyHb. There is little (if any) reaction of GSNO (or CYSNO) at the heme centers of  $\text{Hb}(\text{FeII})\text{O}_2$ . Specifically, the capacity for  $\text{O}_2$  binding to heme is unaffected by RSNOs. See Figure 1D. a. oxyHb. b, c, d. Reaction mixtures of GSNO and oxyHb in ratios of 1:1, 2:1, and 10:1, respectively. Spectra were taken after 60 min of incubation in the spectrophotometer. Further analysis of reaction products revealed the

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formation of SNO-Hb in all cases. Yields of SNO-Hb in spectra *b*, *c* and *d* were 5%, 10% and 50% (S-NO/Hb), respectively. In 5 other determinations, the yield of S-NO/Hb was  $0.37 \pm 0.06$  using GSNO (pH 7.4, 10-fold excess over Hb) and  $\sim 2$  SNO/tetramer ( $1.97 \pm 0.06$ ) using CYSNO (*vida infra*). These last data are in agreement with reports that human HbA contains 2 titratable SH groups.

### Methods

Human HbA<sub>0</sub> was purified from red cells as previously described (Kilbourn, R.G., et al., *Biochem. Biophys. Res. Comm.*, 199:155-162 (1994)). Nitric oxide solutions were rigorously degassed and purified according to standard procedure (Beckman, J. S., et al., *Methods in Nitric Oxide Research*, Feelisch and Stamler, eds., Wiley Chichester, U.K. (1996)) and saturated solutions were transferred in air tight syringes. Deoxygenation of Hb was achieved by addition of excess dithionite (NO studies) or by reduction of Hb(FeII)O<sub>2</sub> through evacuation in Thunberg tubes (RSNO studies; as RSNOs react with dithionite). RSNOs were synthesized as previously described (Gaston, B., et al., (1993); Arnette, D.R. and Stamler, J.S., *Arch. Biochem. Biophys.* 318:270-285 (1995)) Incubations with HbA<sub>0</sub> were made in phosphate buffer, pH 7.4, 0.5 mM EDTA. Quantifications of SNO-Hb were made according to the method of Saville (Gaston, B., et al., (1993); Stamler, J.S., et al., *Proc. Natl Acad. Sci. USA*, 90:444-448 (1992)) after purification of protein with Sephadex G-25 columns. The Saville method, which assays free NO<sub>x</sub> in solution, involves a diazotization reaction with sulfanilamide and subsequent coupling with the chromophore N-(naphthyl)ethylenediamine. No low molecular weight S-NO complexes survived this purification and all activity was protein precipitable.



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The reactions and spectra were carried out using a Perkin Elmer UV/Vis Spectrometer, Lambda 2S.

Example 2: Allosteric Function of O<sub>2</sub> in Regulation of Hb S-nitrosylation

5       Oxygenation of Hb is associated with conformational changes that increase the reactivity of cys $\beta$ 93 to alkylating reagents (Garel, C., et al., *J. Biochem.*, 123:513-519 (1982); Jocelyn, P.C., *Biochemistry of the SH Group*, Academic Press, London, p.243 (1972); Craescu, C.T.,  
10 et al., *J. Biol. Chem.*, 261:14710-14716 (1986)). The physiological importance of this effect has not been explained previously. It was observed here that rates of S-nitrosation of Hb were markedly dependent on conformational state. In the oxy conformation (R state),  
15 S-nitrosation was more rapid than in the deoxy conformation (T state) (Figure 2A). The rate of S-nitrosation was accelerated in both conformations by alkaline conditions (i.e., rate at pH 9.2 > pH 7.4), which tends to expose the cys $\beta$ 93 that is otherwise screened from reaction by the C-  
20 terminal histidine 146 $\beta$ . The salt bridge (asp  $\beta$ 94 --- his  $\beta$ 146) tying down the histidine residue is loosened at high pH. These data suggest that the increase in thiol reactivity associated with the R state derives, at least in part, from improved NO access rather than a conformation-  
25 induced change in pK.

A. Oxygenation accelerates S-nitrosylation of Hb.

Rates of Hb S-nitrosation by S-nitrosocysteine (CYSNO) are faster in the oxy conformation (Hb[FeII]O<sub>2</sub>) than in the deoxy state (Hb[FeII]).

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### Methods

Incubations were performed using 10-fold excess CYSNO over protein (50  $\mu$ M) in aerated 2% borate, 0.5 mM EDTA (oxyHb), or in a tonometer after rapid O<sub>2</sub> evacuation (deoxyHb). At times shown in Figure 2A, samples were rapidly desalted across G-25 columns (preequilibrated with phosphate buffered saline, 0.5 mM EDTA, pH 7.4) to remove CYSNO, and analyzed for SNO-Hb by the method of Saville (Stamler, J.S., et al., *Proc. Natl. Acad. Sci. USA*, 89:444-448 (1992)).

### B. Deoxygenation accelerates denitrosylation of Hb

Rates of RSNO decomposition (and transfer) are much faster in the deoxy conformation [SNO-Hb(FeII)] than in the oxy state [SNO-Hb(FeII)O<sub>2</sub>]. The decomposition of SNO-Hb(FeII) is further accelerated by the presence of excess glutathione. Within the dead time of measurements according to this method (~15 seconds), a major fraction of SNO-Hb(FeII) was converted to GSNO.

### Methods

Hbs in PBS (0.5 mM EDTA, pH 7.4) were incubated in air (oxy) or in a tonometer previously evacuated of O<sub>2</sub> (deoxy). SNO-Hb(FeII)O<sub>2</sub> decomposition was determined by the method of Saville (Saville, B., *Analyst* 83:670-672 (1958)). Spontaneous decomposition of SNO-Hb(FeII) was followed spectrophotometrically by formation of Hb(FeII)NO. Transnitrosation reactions with glutathione were performed by addition of 100-fold excess glutathione over protein (50  $\mu$ M), immediate processing of the reaction mixture under anaerobic conditions followed by rapid TCA precipitation, and analysis of RSNO in the supernatant. Rates of NO group

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transfer were too rapid to measure accurately by the standard methods used in this study.

Example 3: NO-related Interactions with Cysteine Residues of Hb in Physiological Systems

5           Given that Hb is largely contained within red blood cells, potential mechanisms by which S-nitrosation of intracellular protein might occur were explored. Incubation of oxygenated rat red blood cells with S-nitrosocysteine resulted in very rapid formation of  
10 intracellular SNO-Hb(FeII)O<sub>2</sub> (Figure 3A). Rapid oxidation of Hb was not observed under these conditions. Intraerythrocytic SNO-Hb also formed when red blood cells were treated with S-nitrosohomocysteine or S-nitrosocysteinyglycine, but not with S-nitrosoglutathione  
15 (GSNO). Thus, erythrocyte access of RSNOs is thiol group specific. Exposure of oxygenated red blood cells to NO resulted primarily in methHb formation.

Endothelium-derived relaxing factor (EDRF) and hemoglobin

Hb-mediated inhibition of endothelium-dependent  
20 relaxations is commonly used as a marker of NO responses. Inasmuch as reactions with either metal or thiol centers of Hb should lead to attenuated NO/EDRF (endothelium-derived relaxing factor) responses, experiments were performed to elucidate the molecular basis of inhibition. Hb  
25 preparations in which  $\beta$ 93 thiol groups had been blocked with N-ethylmaleimide (NEM) or the hemes blocked by cyanmet (FeIIICN)-derivitization were studied in an aortic ring bioassay, and their activities were compared with that of native Hb. Both cyanmet-Hb and NEM-Hb caused increases in  
30 vessel tone and attenuated acetylcholine (EDRF)-mediated relaxations (Figure 3B). However, native Hb was

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significantly more effective than either of the modified Hb preparations (Figure 3B). Taken in aggregate, these studies suggest that both the thiol and metal groups of Hb contribute to its NO-related activity. To verify formation of an S-nitrosothiol in Hb, a bioassay was used in which 2 cm segments of thoracic aorta were interposed in Tygon tubing, through which 3 cc of Krebs solution containing Hb (4  $\mu$ M) and ACh (2  $\mu$ M) were circulated by roller pump (1.5 cc/min x 5 min). Analysis of the effluent (Gaston, B., et al., (1993)) revealed the formation of SNO-Hb (20  $\pm$  4 nM) in 5 of 5 experiments.

A. S-nitrosation of intraerythrocytic Hb

Incubation of rat erythrocytes with S-nitrosocysteine (equimolar to heme (5 mM); phosphate buffer pH 7.4, 25°C) leads to rapid formation of intracellular SNO-Hb(FeII)O<sub>2</sub>. MetHb does not form rapidly. Separation of intracellular RSNOs across G-25 columns reveals that only a small percentage exists as low molecular weight S-nitrosothiol (e.g. GSNO) at most time points. By 60 min, 3 of the 4 available SH groups of Hb were S-nitrosated (note that rat Hb contains 4 reactive SH groups). See Figure 3A. Inset shows spectra of SNO-Hb isolated from rat erythrocytes and related analyses. Spectrum A is that of SNO-Hb isolated from erythrocytes following G-25 chromatography. Treatment of A with dithionite results in reduction of the S-NO moiety, liberating free NO which is autocaptured by deoxy Hb, forming Hb(FeII)NO (note that dithionite simultaneously deoxygenates Hb) (spectrum C). This spectrum (C) reveals a stoichiometry of ~3 S-NOs per tetramer. The spectrum of

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Hb(FeII)NO containing 4 NO's per tetramer is shown for comparison (inset, spectrum B).

### Methods

At shown intervals, red blood cells were pelleted  
5 rapidly by centrifugation, washed three times, lysed in  
deionized water at 4°C, and the cytosolic fraction  
subjected to rapid desalting across G-25 columns.  
Intracellular SNO-Hb was measured by the method of Saville  
(Gaston, B., et al., (1992); Stamler, J.S., et al., *Proc.*  
10 *Natl. Acad. Sci. USA*, 89:444-448 (1992)), and confirmed  
spectroscopically (inset of Figure 3A) as described above.

### B. Molecular Basis of EDRF/Hb Interaction.

The effects of native Hb on EDRF responses were  
compared with Hb preparations in which the thiol or heme  
15 centers had been blocked by alkylation or cyanmet  
derivitization, respectively. All preparations of Hb  
elicited contractions; however, those of native Hb (in  
which both SH and metal centers are free for interaction)  
were most pronounced. See Figure 3B. Likewise,  
20 acetylcholine (ACh) mediated relaxations were most  
effectively inhibited by native Hb. Relaxations were  
inhibited to lesser degrees by cyanmet Hb (CN-Hb) (in which  
hemes were blocked from reaction) and NEM-Hb (in which  
thiol groups were alkylated by N-ethylmaleimide). See  
25 Table 1. These data illustrate that both heme and  $\beta$ 93SH  
groups of Hb contribute to reversal of EDRF responses.  
Direct measurement of SNO-Hb, formed from EDRF under  
similar conditions, is described in Example 8.

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Methods

Descending rabbit thoracic aorta were cut into 3 mm rings and mounted on stirrups attached to force transducers (model FT03, Grass Instruments, Quincy, MA) for measurement of isometric tone. The details of this bioassay system have been previously described (Stamler, J.S., et al., *Proc. Natl. Acad. Sci. USA*, 89:444-448 (1992)). Cyanmet Hb was prepared from human HbA according to published protocols (Kilbourn, R.G. et al. *Biochem. Biophys. Res. Comm.*, 199:155-162, (1994)). Alkylation of HbA with N-ethylmaleimide was followed by desalting across G-25 Sephadex to remove excess NEM. Removal of unmodified Hbcys $\beta$ 93 was achieved by passage through Hg-containing affinity columns. The alkylation of free SH groups was verified using 5,5'-dithio-bis[2-nitrobenzoic acid].

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TABLE 1

ADDITIONS	% INCREASE IN TENSION	% ACh RELAXATION
Hb (1 $\mu$ M)	40.8 $\pm$ 2.3 (n=7)	31.9 $\pm$ 6.9 (n=7)
NEM-Hb (1 $\mu$ M)	29.4 $\pm$ 1.3** (n=7)	60.5 $\pm$ 3.9* (n=7)
CN-Hb (1 $\mu$ M)	12.9 $\pm$ 3.0** (n=6)	80.7 $\pm$ 1.0** † (n=4)
ACh (1 $\mu$ M)		98.3 $\pm$ 0.6 (n=10)
*, P<0.01; **, P<0.001, Compared to Hb; †, P<0.001, Compared to ACh		

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Example 4: Transduction of SNO-Hb Vasoactivity

Arterial red blood cells contain two physiologically important forms of hemoglobin:  $\text{Hb(FeII)O}_2$  and  $\text{Hb(FeIII)}$  (Antonini, E. and Brunori, M. *In Hemoglobin and Myoglobin in Their Reactions with Ligands*, American Elsevier Publishing Co., Inc., New York, pp. 29-31 (1971)).

Arterial-venous differences in the S-nitrosothiol content of intraerythrocytic Hb suggest that the NO group is released during red cell transit. Such findings raise the possibility of functional consequences, perhaps influenced by the redox state of heme and its occupation by ligand.

$\text{SNO-Hb(FeII)O}_2$  was found to possess modest NO-like activity when tested in a vascular ring bioassay. Specifically, the contraction elicited by  $\text{SNO-Hb(FeII)O}_2$  was less than that of native  $\text{Hb(FeII)O}_2$ , indicating that S-nitrosation partially reverses the contractile effects of Hb (Figure 4A). By comparison,  $\text{SNO-Hb(FeIII)}$  was found to be a vasodilator (Figure 4A). Notably, free NO was devoid of relaxant activity in the presence of  $\text{Hb(FeII)O}_2$  or  $\text{Hb(FeIII)}$ .

Red blood cells contain millimolar concentrations of glutathione. As equilibria among RSNOs are rapidly established through RSNO/thiol exchange (Arnette, D.R. and Stamler, J.S., *Arch. Biochem. Biophys.*, 318:279-285 (1995)), the vasoactivity of SNO-Hb was reassessed in the presence of glutathione. Figure 4B illustrates that glutathione potentiated the vasodilator activity of both  $\text{SNO-Hb(FeII)O}_2$  and  $\text{SNO-Hb(FeIII)}$ . GSNO formation under these conditions (confirmed chemically and in bioassay experiments) appeared to fully account for this effect. Further kinetic analyses revealed that transnitrosation involving glutathione was more strongly favored in the equilibrium with  $\text{SNO-Hb(FeIII)}$  than  $\text{SNO-Hb(FeII)O}_2$  (Figure 4C). Given the findings of



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steady-state levels of SNO-Hb in red blood cells (Table 2 and Figure 3A), these results suggest that 1) the equilibrium between naturally occurring RSNOs and Hb(cys $\beta$ 93) lies toward SNO-Hb under physiological conditions; 2) that transnitrosation reactions involving SNO-Hb and GSH are likely to occur within red blood cells (in these studies, low molecular weight RSNOs have been found in erythrocytes loaded with SNO-Hb); and 3) that oxidation of the metal center of Hb shift the equilibrium toward GSNO, thereby potentially influencing bioactivity.

Additional mechanisms of NO group release from SNO-Hb were sought. Arterial-venous differences in levels of SNO-Hb raised the possibility that S-NO bond stability may be regulated by the changes in Hb conformation accompanying deoxygenation. To test this possibility, the rates of NO group release from SNO-Hb(FeII)O<sub>2</sub> and SNO-Hb(FeIII) were compared. Deoxygenation was found to enhance the rate of SNO-Hb decomposition (Figure 2B). These rates were accelerated greatly by glutathione in a reaction yielding GSNO (Figure 2B). The results illustrate that O<sub>2</sub>-metal interactions influence S-NO affinity, and suggest a new allosteric function for Hb.

For SNO-Hb to be of physiological importance it must transduce its NO-related activity across the erythrocyte membrane. This possibility was explored by incubating erythrocytes containing SNO-Hb in physiologic buffer, and measuring the accumulation of extracellular RSNOs over time. Figure 4D illustrates that red blood cells export low molecular weight (trichloroacetic acid soluble) S-nitrosothiols under these conditions. Importantly, the degree of hemolysis in these experiments was trivial (<0.5%), and correction for lysis did not significantly impact on rates of RSNO release. These results establish

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that an equilibrium exists between low molecular weight and protein RSNOs within the red cell, and that intracellular location is unlikely to be a limiting factor in the transduction of such NO-related activity to the vessel wall.

A. Concentration-effect responses of different SNO-Hb preparations

Contractile effects of Hb(FeII)O<sub>2</sub>(▲) are shown to be partially reversed by S-nitrosation (SNO-Hb[FeII]O<sub>2</sub>(■); P = 0.02 by ANOVA vs Hb(FeII)O<sub>2</sub>) (See Figure 4A.). Oxidation of the metal center of SNO-Hb (SNO-Hb[FeIII](●)) converts the protein into a vasodilator (P < 0.0001 by ANOVA vs. SNO-Hb[FeII]O<sub>2</sub>), with potency comparable to that of other S-nitrosoproteins (Stamler, J.S., et al., *Proc. Natl. Acad. Sci. USA*, 89:444-448 (1992)). The contractile properties of Hb(FeIII) are shown for comparison (□); n = 6-17 for each data point.

Methods

Details of the vessel ring bioassay have been published (Stamler, J.S., et al., *Proc. Natl. Acad. Sci. USA* 89:444-448 (1992)). SNO-Hb(FeII)O<sub>2</sub> preparations were synthesized with 10-fold excess S-nitrosocysteine (CYSNO) over Hb(FeII)O<sub>2</sub> protein (2% borate, 0.5 mM EDTA, ~15 min incubation), after which desalting was performed across Sephadex G-25 columns. CYSNO was synthesized in 0.5 N HCl, 0.5 mM EDTA and then neutralized (1:1) in 1 M phosphate buffer containing 0.5 mM EDTA. SNO-Hb(FeIII) preparations followed a similar protocol, but used Hb(FeIII) as starting material. The latter was synthesized by treatment of Hb(FeII)O<sub>2</sub> with excess ferricyanide, followed by desalting across G-25 columns. SNO-Hb concentrations were verified

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spectroscopically and the S-nitrosothiol content was determined by the method of Saville (Stamler, J.S., et al., *Proc. Natl. Acad. Sci USA* 89:444-448 (1992)). The S-NO/tetramer stoichiometry for both SNO-Hb preparations was ~2. Oxidation of the heme was undetectable by uv-spectrophotometric methods.

B. Potentialiation of SNO-Hb effects by glutathione

Addition of glutathione (100  $\mu$ M) to bioassay chambers potentiates the dose-response to both SNO-Hb(FeII)O<sub>2</sub> (■) and SNO-Hb(FeIII) (●) (See Figure 4B. n = 6-12; p < 0.0001 for both by ANOVA, compared with the respective tracings in Figure 4A. Glutathione had a transient affect on baseline tone in some experiments, and did not significantly influence the response to Hb(FeII)O<sub>2</sub> (▲).

C. Transnitrosation between SNO-Hb and glutathione

Rates of NO group transfer from SNO-Hb (100  $\mu$ M) to glutathione (10 mM) are displayed for SNO-Hb(FeII)O<sub>2</sub> (oxy) and SNO-Hb(FeIII) (met) (n=5). Data are presented as the amount of GSNO formed relative to the starting SNO-Hb concentration. The transfer is more rapid for SNO-Hb(FeIII) than SNO-Hb(FeII)O<sub>2</sub> (p<0.002 by ANOVA), suggesting that the GSNO/SNO-Hb equilibrium is shifted toward GSNO by formation of metHb.

Methods

Thiol/SNO-Hb exchange, forming GSNO, was verified chemically (Stamler, J.S., et al., *Proc. Natl Acad. Sci. USA*, 89:444-448 (1992)) following trichloroacetic acid precipitation (n=5). These results were verified in separate experiments by measuring the residual SNO-Hb

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concentration, following separation of reaction mixtures across G-25 columns.

D. Export of S-nitrosothiols by red blood cells

Human red blood cells containing SNO-Hb are shown to export low molecular weight RSNOs over time. Hemolysis, which ranged from 0- $<0.5\%$  over one hour and did not correlate with rates of RSNO release, could account for only a trivial fraction of the measured extracellular RSNO.

Methods

Packed human red blood cells were obtained by centrifugation, washed, and resuspended in phosphate buffered saline containing 5 mM SNOCYS (0.5 mM EDTA, pH 7.4) for one hour. This results in a red cell preparation containing SNO-Hb (FeII $O_2$ /FeIII mixture) with a stoichiometry of 0.5 S-NO/tetramer. The red blood cells were then washed repeatedly to remove residual CYSNO (verified), and incubated in Krebs' solution (1:4). The accumulation of extracellular RSNO was measured over time by the method of Saville (Saville, B., *Analyst*, 83:670-672 (1958)). Hemolysis was determined by spectral analysis of red blood cell supernatants following centrifugation.

Example 5: SNO-Hb Bioactivity In Vivo

Systemic administration of cell-free Hb results in hypertensive responses which have been attributed to NO scavenging by the heme (Vogel, W.M., et al., *Am. J. Physiol.* 251:H413-H420 (1986); Olsen, S.B., et al., *Circulation* 93:329-332 (1996)). To determine if SNO-Hb is free of this adverse affect, and to explore if in vitro mechanisms of NO release extend to the in vivo circumstance, we compared responses to Hb and SNO-Hb

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infused as a bolus into the femoral vein of anesthetized rats. As illustrated in Figure 5, Hb(FeII)O<sub>2</sub> (200 nmol/kg) caused an increase in mean arterial pressure of  $20 \pm 3$  mm Hg ( $n = 4$ ;  $P < 0.05$ ). In contrast, SNO-Hb(FeII)O<sub>2</sub> did not exhibit hypertensive effects and SNO-Hb(FeIII) elicited hypotensive responses (Figure 5). Thus, the profiles of these compounds *in vivo* closely resemble those seen *in vitro* (Figure 4A). Moreover, to demonstrate that the physiological responses of red cells are comparable to those of cell-free Hb preparations, erythrocytes containing SNO-Hb were injected into the femoral vein of rats pretreated with L-NMMA (50 mg/kg) to deplete endogenous RSNOs. At levels of SNO-Hb comparable to those found in the normal rat (0.1-0.5  $\mu$ M), SNO-Hb containing red blood cells elicited hypotensive responses ( $8 \pm 1$  mm Hg; mean  $\pm$  SEM;  $n=9$ ), whereas native (SNO-Hb depleted) red blood cells did not ( $P=0.001$ ). These changes in mean blood pressure of ~10% are on the order of those that differentiate normotension from hypertension in man, and in the therapeutic range of some antihypertensive regimens. The effects of both Hb and SNO-Hb -- whether cell-free or contained within red cells -- were transient, suggesting that S-nitrosylation of Hb and metabolism of SNO-Hb is occurring *in vivo*, with consequent restoration of blood pressure. The bioactivity of SNO-Hb in blood, where S-NO/heme stoichiometries approach 1:50,000, is a dramatic illustration of the resistance of this NO-related activity to Hb(Fe) inactivation.

#### In vivo effects of cell-free Hb and SNO-Hbs

Administration of 2-200 nmol/kg Hb(FeII)O<sub>2</sub> (as a bolus) into the femoral vein of a Sprague-Dawley rat is shown to increase mean arterial pressure in a dose-

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dependent manner. At 200 nmol/kg, mean arterial pressure increased by 25 mm Hg ( $20 \pm 3$  mm Hg;  $n = 4$ ;  $P < 0.05$ ). Elevations in blood pressure reversed within 10-15 minutes. SNO-Hb(FeII)O<sub>2</sub> infusions (over the same dose range) are shown to ameliorate Hb(FeII)O<sub>2</sub>-induced hypertension without causing overt changes in blood pressure. A similar response was seen at higher doses. By comparison, SNO-Hb(FeIII) infusions caused a significant fall in mean arterial pressure (pre  $108 \pm 4$  mm Hg; post  $74 \pm 6$  mm Hg,  $n=5$ ;  $P < 0.05$ ) at the highest dose (200 nmol/kg). Hypotensive responses tended to be transient with blood pressure normalizing over 10 minutes. A fall in blood pressure was also seen with injection of erythrocytes containing SNO-Hb.

15

#### Methods

Rats were anesthetized by intraperitoneal injection of pentobarbital and the femoral arteries and veins accessed by local cut down. The artery was then cannulated and the blood pressure monitored continuously using a Viggo Spectramed pressure transducer attached to a Gould recorder. An IBM PC (DATA Q Codas) was used for data acquisition.

Example 6: Loading of Red Blood Cells With S-Nitrosothiols

Incubation of rat erythrocytes with S-nitrosocysteine (equimolar to heme (5 mM); phosphate buffer pH 7.4, 25°C) leads to rapid formation of intracellular S-nitrosothiols. MetHb does not form rapidly. Separation of cell content across G-25 columns establishes the formation of intraerythrocytic low molecular weight S-nitrosothiol, e.g. S-nitrosogluthathione, (GSNO). By 2 minutes, one can achieve as much as millimolar GSNO.

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Method for assay of RSNO

S-nitrosocysteine (5 mM) treated red blood cells are pelleted rapidly by centrifugation, washed three times, lysed in deionized water at 4°C, and the cytosolic fraction  
5 subjected to rapid desalting across G-25 columns. Intracellular RSNO is measured by the method of Saville and can be confirmed spectroscopically.

Effects on blood pressure from loaded red blood cells

Red blood cells treated with S-nitrosocysteine (to produce SNO-RBCs) and introduced into the femoral vein of a Sprague-Dawley rat decreased mean arterial pressure in a  
5 dose-dependent manner. For red blood cells in which SNO-Hb was assayed at 0.3  $\mu$ M (the endogenous in vivo SNO-Hb concentration), arterial pressure decreased by  $8 \pm 1$  mm Hg (mean  $\pm$  SEM for 9 experiments;  $p < 0.001$  compared to untreated red blood cell controls). For red blood cells in  
10 which SNO-Hb was assayed at 0.5  $\mu$ M, arterial pressure decreased by 10 mm Hg. For red blood cells in which SNO-Hb was assayed at 0.1  $\mu$ M (a sub-endogenous SNO-Hb concentration), arterial pressure decreased by 6 mm Hg. The administration of untreated red blood cells caused no  
15 effect or a slight increase in arterial blood pressure. Administration of L-monomethyl-L-arginine (L-NMMA; 50 mg/kg) caused an increase in blood pressure of about 20 mm Hg. Changes in blood pressure from a bolus administration of loaded red blood cells lasted 15-20 minutes.

20 Further methods

Rats were anesthetized by intraperitoneal injection of pentobarbital and the femoral arteries and veins accessed by local cut down. The artery was then cannulated and the blood pressure monitored continuously using a Viggo

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Spectramed pressure transducer attached to a Gould recorder. An IBM PC (DATA Q Cudas) was used for data acquisition.

Example 7: Effects of SNO-Hb on Coronary Vasodilation,

5 Coronary Flow and Blood Pressure

SNO-Hb was synthesized as described in Example 4A. Completion of the reaction was determined as described in Example 4A. Twenty-four healthy mongrel dogs (25-30 kg) were anesthetized with intravenous thiamylal sodium (60-80  
10 mg/kg) and subjected to left thoracotomy in the fourth intercostal space. The left circumflex coronary artery distal to the left atrial appendage was minimally dissected. A pair of 7-MHz piezoelectric crystals (1.5 X 2.5 mm, 15-20 mg) was attached to a Dacron backing and  
15 sutured to the adventitia on opposite surfaces of the dissected vessel segment with 6-0 prolene. Oscilloscope monitoring and on-line sonomicrometry (sonomicrometer 120-2, Triton Technology, San Diego, CA) were used to ensure proper crystal position. A pulse Doppler flow probe (10  
20 MHz, cuff type) was implanted distal to the crystals. An inflatable balloon occluder was also placed distal to the flow probe. All branches of the circumflex artery between the crystals and the occluder were ligated. Heparin sodium-filled polyvinyl catheters were inserted into the  
25 left ventricular cavity via the apex, into the left atrium via the atrial appendage, and into the ascending aorta via the left internal thoracic artery. The catheters, tubing, and wires were tunnelled to a subcutaneous pouch at the base of the neck.

30 After a 10 to 15 day recovery period, the catheters and wires were exteriorized under general anesthesia, and 2-3 days later, each dog was given a bolus injection of



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SNO-Hb (0.4 mg) to evaluate vascular response. Two dogs that demonstrated <5% dilation of epicardial coronary vessels were excluded from subsequent studies, and two were excluded because of other technical reasons.

5        Dogs were trained and studied while loosely restrained and lying awake in the lateral recumbent position. The laboratory was kept dimly illuminated and quiet. Aortic pressure, left ventricular end-diastolic pressure dP/dt external coronary diameter and coronary flow were monitored  
10 continuously. In 10 dogs, 0.1 ml of SNO-Hb solution, 50 nM/kg, was injected via the left atrial catheter. To verify potential effects of solvent on vasculature, 0.1 ml injections of 30% ethanol in distilled water were given as vehicle control. Between injections, phasic coronary blood  
15 flow and coronary artery diameter were allowed to return to preinjection levels (minimum 15 minutes). Allowing a 15 minute period between injections resulted in no modification of repeated does injections. To assess the direct and potential flow mediated indirect vasodilation  
20 effects of SNO-Hb on the conductance vessels, the dose was repeated in 6 of 10 dogs with partial inflation of the adjustable occluder to maintain coronary blood flow at or slightly below preinjection levels. The response to acetylcholine chloride (Sigma Chemical) was assessed in  
25 another group of 10 dogs following a similar protocol to that used for SNO-Hb.

      Epicardial coronary diameter, coronary blood flow, heart rate, and aortic and left ventricular end-diagnostic pressures were compared before and after each SNO-Hb  
30 injection. The maximum changes in coronary dimension and blood flow were expressed as a function of increasing doses of SNO-Hb. The response of coronary dimension to increasing doses followed a characteristic sigmoid dose-

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response curve that could be described by the following equation

$$Effect = \frac{maximal\ effect \times\ dose}{K_D +\ dose}$$

where  $K_D$  is the drug-receptor complex dissociation constant and is the dose at which 50% of the maximum response ( $EC_{50}$ ) is achieved. In each animal, a nonlinear least-squares regression ( $r^2 > 0.90$ ) was performed on the dose-response data. The regression was constrained to the above equation. From the regression, values for maximum response and  $K_D$  were obtained for each individual animal. The mean of these values was then calculated to obtain an average  $K_D$  and maximum response for the study group. These values were used to generate a mean curve, which was plotted with the mean dose-response values. (See Figures 6A-6F.)

Example 8: Endogenous Levels of S-nitrosohemoglobin and Nitrosyl(FeII)-Hemoglobin in Blood

To determine if SNO-Hb is naturally occurring in the blood, and if so, its relationship to the  $O_2$  transport capacity and nitrosylated-heme content of red cells, an analytical approach was developed to assay the S-nitrosothiol and nitrosyl-heme content of erythrocytes (Table 2). Arterial blood was obtained from the left ventricle of anesthetized rats by direct puncture and venous blood was obtained from the jugular vein and inferior vena cava. Hb was then purified from red cells and assayed for RSNO and (FeII)NO content. Arterial blood contained significant levels of SNO-Hb, whereas levels were virtually undetectable in venous blood (Table 2).

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Measurements made 45 minutes after infusion of the NO synthase inhibitor N<sup>ω</sup>-monomethyl-L-arginine (L-NMMA) (50 mg/kg), showed a depletion of SNO-Hb as well as total Hb-NO (82 and 50 ± 18%, respectively; n = 3-5; p < 0.05). These data establish the endogenous origin of SNO-Hb, although some environmental contribution is not excluded. The arterial-venous distribution seen for SNO-Hb was reversed in the case of Hb(FeII)NO, which was detected in higher concentrations in partially deoxygenated (venous) erythrocytes (Table 2). Accordingly, the proportion of nitrosylated protein thiol and heme appears to depend on the oxygenation state of the blood. Consistent with these findings, Wennmalm and coworkers have shown that Hb(FeII)NO forms mainly in venous (partially deoxygenated) blood (Wennmalm, A., et al., *Br. J. Pharmacol.* 106(3):507-508 (1992)). However, levels of Hb(FeII)NO *in vivo* are typically too low to be detected (by EPR) and SNO-Hb is EPR-silent (*i.e.*, it is not paramagnetic). Thus, photolysis-chemiluminescence represents an important technological advance, as it is the first methodology capable of making quantitative and functional assessments of NO binding to Hb under normal physiological conditions.

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Table 2

Endogenous Levels of S-Nitrosohemoglobin and  
Nitrosyl(FeII)-Hemoglobin in Blood

Site	SNO-Hb (nM)	Hb(FeII)NO (nM)
Arterial	311 ± 55*	536 ± 99 †
Venous	32 ± 14	894 ± 126

\*P < 0.05 vs venous; † P < 0.05 for paired samples vs venous

### Methods

Blood was obtained from the left ventricle (arterial) and jugular vein (venous) of anesthetized Sprague-Dawley rats. Comparable venous values were obtained in blood from the inferior vena cava. Red blood cells were isolated by centrifugation at 800 g, washed three times in phosphate buffered saline at 4°C, lysed by the addition of 4-fold excess volume of deionized water containing 0.5 mM EDTA, and desalted rapidly across G-25 columns according to the method of Penefsky at 4°C. In 24 rats, Hb samples were divided in two aliquots which were then treated or not treated with 10-fold excess HgCl<sub>2</sub> over protein concentration as measured by the method of Bradford. Determinations of SNO-Hb and Hb(FeII)NO were made by photolysis-chemiluminescence. In 12 additional rats, further verification of the presence of SNO-Hb was made by assaying for nitrite after HgCl<sub>2</sub> treatment. Specifically, samples (with and without HgCl<sub>2</sub>) were separated across Amicon-3 (Centricon filters, m.w. cut off 3,000) at 4°C for

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1 h, and the low molecular weight fractions collected in airtight syringes containing 1  $\mu$ M glutathione in 0.5 N HCl. Under these conditions, any nitrite present was converted to S-nitrosoglutathione, which was then measured by photolysis-chemiluminescence (detection limit  $\sim$ 1 nM). SNO-Hb was present in all arterial samples, and levels determined by this method ( $286 \pm 33$  nM) were virtually identical to and not statistically different from those shown in Table 2. In venous blood, SNO-Hb was undetectable (0.00  $\pm$  25 nM); levels were not statistically different from those given above.

#### Method for assay of S-nitrosohemoglobin

A highly sensitive photolysis-chemiluminescence methodology was employed. A somewhat similar assay has been used for measuring RSNOs (S-nitrosothiols) in biological systems (Gaston, B., et al., *Proc. Natl. Acad. Sci. USA* 90:10957-10961 (1993); Stamler, J.S., et al., *Proc. Natl. Acad. Sci. USA* 89:7675-7677 (1992)). The method involves photolytic liberation of NO from the thiol, which is then detected in a chemiluminescence spectrometer by reaction with ozone. The same principle of operation can be used to cleave (and measure) NO from nitrosyl-metal compounds (Antonini, E. and Brunori, M. In *Hemoglobin and Myoglobin in Their Reactions with Ligands*, American Elsevier Publishing Co., Inc., New York, pp. 29-31 (1971)). With adjustment of flow rates in the photolysis cell, complete photolysis of the NO ligand of Hb(FeII)NO could be achieved. Standard curves derived from synthetic preparations of SNO-Hb, Hb(FeII)NO, and S-nitrosoglutathione were linear ( $R > 0.99$ ), virtually superimposable, and revealing of sensitivity limits of

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approximately 1 nM. Two analytical criteria were then found to reliably distinguish SNO-Hb from Hb(FeII)NO:

- 1) signals from SNO-Hb were eliminated by pretreatment of samples with 10-fold excess  $\text{HgCl}_2$ , while Hb(FeII)NO was
- 5 resistant to mercury challenge; and 2) treatment of SNO-Hb with  $\text{HgCl}_2$  produced nitrite (by standard Griess reactions) in quantitative yields, whereas similar treatment of Hb(FeII)NO did not. UV/VIS spectroscopy confirmed that NO remained attached to heme in the presence of excess  $\text{HgCl}_2$ .

10 Example 9: Inhibition of Platelet Aggregation by S-Nitrosohemoglobin

Methods to prepare human HbA<sub>0</sub> were as described in Example 1 "Methods" section. Methods to make SNO-Hb(FeII)O<sub>2</sub> were as described for Example 2A. Methods to

15 make SNO-Hb(FeIII) were as in Example 1 (see parts B, C, and "Methods" in Example 1). Quantifications of SNO-hemoglobins were made as in Example 1 according to the method of Saville (Saville, B., *Analyst* 83:670-672 (1958)) and by the assay as described in Example 8, "Method for

20 assay of S-nitrosohemoglobin."

Venous blood, anticoagulated with 3.4 nM sodium citrate, was obtained from volunteers who had not consumed acetylsalicylic acid or any other platelet-active agent for at least 10 days. Platelet-rich plasma was prepared by

25 centrifugation at 150 x g for 10 minutes at 25°C and was used within 2 hours of collection. Platelet counts were determined with a Coulter counter (model ZM) to be 1.5 to 3 x 10<sup>8</sup>/ml.

Aggregation of platelet-rich plasma was monitored by a

30 standard nephelometric technique in which results have been shown to correlate with bleeding times. Aliquots (0.3 ml) of platelets were incubated at 37°C and stirred at 1000 rpm

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in a PAP-4 aggregometer (Biodata, Hatsboro, PA).

Hemoglobins were preincubated with platelets for 10 min and aggregations were induced with 5  $\mu$ M ADP. Aggregations were quantified by measuring the maximal rate and extent of

5. change of light transmittance and are expressed as a normalized value relative to control aggregations performed in the absence of hemoglobin.

The results of the aggregation assays are shown in Figures 7A, 7B and 7C. Standard deviations are shown as  
10 vertical bars. SNO-Hb[Fe(II)]O<sub>2</sub> causes some inhibition of platelet aggregation at the higher concentrations tested. SNO-Hb[Fe(III)] also inhibits platelet aggregation when present at concentrations of 1  $\mu$ M and above, but to a much greater extent than SNO-Hb[Fe(II)]O<sub>2</sub>.

15. Example 10: Effect of SNO-Hbs on cGMP

Platelet rich plasma (PRP) was incubated with either hemoglobin, SNO-oxy Hb, or SNO-metHb for 5 min, after which the assay was terminated by the addition of 0.5 ml of ice cold trichloroacetic acid to 10%. Ether extractions of the  
20 supernatant were performed to remove trichloroacetic acid, and acetylation of samples with acetic anhydride was used to increase the sensitivity of the assay. Measurements of cyclic GMP were performed by radioimmunoassay (Stamler, J. et al., *Circ. Res.* 65:789-795 (1989)).

- 25 Results are shown in Figure 8. For all concentrations of Hb tested (1, 10 and 100  $\mu$ M), the concentration of cGMP measured for SNO-Hb(FeIII) was less than that of native Hb.

Example 11: Polynitrosation of Hb

- A. HbA<sub>0</sub> (oxy) was incubated with S-nitrosoglutathione at  
30 a ratio of 6.25 S-nitrosoglutathione/HbA<sub>0</sub> for 240 minutes at pH 7.4 at 25°C and desalted over Sephadex G-25 columns.

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Spectra were run in the presence (spectrum B, Figure 9A) and absence (spectrum A, Figure 9A) of dithionite. The shift in the spectrum is indicative of 2 SNO groups/tetramer.

- 5 B. HbA<sub>0</sub> was incubated with 100-fold excess S-nitrosogluthathione over protein for 240 minutes at pH 9.2, followed by desalting over a G-25 column. A portion was then treated with dithionite. The spectra in Figure 9B indicate that Hb has been nitrosated at multiple sites.
- 10 C. HbA<sub>0</sub> was treated with 100-fold excess S-nitrosocysteine over tetramer at pH 7.4, 25°C for 5-20 min. After various times of treatment, the protein was desalted over a G-25 column and treated with dithionite. The spectra show progressive polynitrosation of Hb with time (spectra A to F
- 15 in Figure 9C). After 5 minutes of treatment with 100-fold excess S-nitrosocysteine, 0.09 NO groups had added per tetramer (spectrum A of Figure 9C); after 20 minutes, at least 4 NO groups had added (spectrum F). At intermediate time points, 0.4 NO groups (spectrum B), 1.58 NOs (spectrum
- 20 C), 2.75 NOs (spectrum D) or 2.82 NOs had added per tetramer (spectrum E).

- D. Rat Hb was treated with 100x S-nitrosogluthathione excess over tetramer for 3 hours at pH 7.4. The protein was then desalted by passage through a G-25 column. A
- 25 portion of the desalted protein was treated with dithionite (spectrum B in Figure 9D; the protein of spectrum A was left untreated by dithionite). Spectrum B in Figure 9D is illustrative of a ratio of 6 RNOS/Hb.



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- E. A time course experiment tracking the extent of nitrosation of HbA<sub>0</sub> with time was performed (Figure 9E). Treatment of HbA<sub>0</sub> was with 10x excess S-nitrosocysteine at pH 7.4, 25°C or with 100x excess S-nitrosocysteine under the same conditions. Analysis for SNO and NO was performed by the method of Saville and by UV spectroscopy as in Jia, L. et al., *Nature* 380:221-226 (1996). Under these conditions the heme is ultimately oxidized; the rate is time dependent.
- 10 Treatment with 10x excess S-nitrosocysteine nitrosylates only the thiol groups of the two reactive cysteine residues of HbA<sub>0</sub>. Inositol hexaphosphate is known to shift the allosteric equilibrium towards the T-structure (ordinarily, the deoxy form). Treatment with 100x excess
- 15 nitrosates additional groups; i.e., the product has more than 2 NO groups/tetramer.

Example 12: Effect of SNO-Hb(FeII)O<sub>2</sub> on Blood Flow

- SNO-Hb(FeII)O<sub>2</sub>, having a SNO/Hb ratio of 2, was prepared (from HbA<sub>0</sub>) by reaction with S-nitrosothiol. Rats breathing 21% O<sub>2</sub> were injected (time 0) with Hbs prepared from HbA<sub>0</sub> as indicated in Figure 10 (open circles, SNO-Hb (100 nmol/kg); filled circles, SNO-Hb (1000 nmol/kg); filled squares, unmodified Hb (1000 nmol/kg)). Three rats were used per experiment. Blood flow was measured in brain
- 20 using the H<sub>2</sub> clearance method; microelectrodes were placed in the brain stereotactically. Concomitant PO<sub>2</sub> measurements revealed tissue PO<sub>2</sub> = 20. Thus, SNO-Hb improves blood flow to the brain under normal physiological conditions, whereas native Hb decreases blood flow. NO
- 25 group release is promoted by local tissue hypoxia.
- 30

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Example 13: Effects of SNO-Hb(FeII)O<sub>2</sub>, SNO-Hb(FeIII) and (NO)<sub>x</sub>-Hb(FeIII) on Tension of Rabbit Aorta

Hemoglobin was treated with either 1:1, 10:1 or 100:1 S-nitrosocysteine to Hb tetramer for 1 hour, processed as  
5 in Example 4. The products of the reactions done with 1:1 and 10:1 excess were assayed by the Saville assay and by standard spectrophotometric methods. The product of the reaction done at the 1:1 ratio is SNO-Hb(Fe)O<sub>2</sub>; SNO-Hb(FeIII) is found following reaction with 1:10 excess  
10 CYSNO/tetramer.

The aortic ring bioassay was performed as described in Example 4. The product of the reaction in which a ratio of 100:1 CYSNO/Hb tetramer was used, contains 2 SNOs as well as NO attached to the heme. The potency of the 100:1  
15 CYSNO/Hb product is much greater than that of SNO-Hb(FeIII) and is indicative of polynitrosation (see Figure 11).

Example 14: Effect of Oxygenation on Partially Nitrosylated Hemoglobin

The effect of oxygenation on partially nitrosylated Hb  
20 was examined by following spectral changes in the Soret region upon the addition of air to partially nitrosylated Hb. Hemoglobin A (17 μM) was deoxygenated by bubbling argon through a 1 ml solution in 100 mM phosphate (pH 7.4), for 45 minutes. Nitric oxide was added by injection of 0.5  
25 μl of a 2 mM solution, stored under nitrogen. The final heme:NO ratio was 68:1. The solution was slowly aerated by sequential 50 μl injections of room air. Figure 12 shows that the initial additions of air failed to produce a true isosbestic point, indicating changes in the concentrations  
30 of at least three absorbent species. Later additions of air did produce a true isosbestic point, indicative of the conversion of deoxyhemoglobin to oxyhemoglobin, with the

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loss of nitrosyl heme. The results show that nitrosylated Hb is not a stable end product.

Example 15: Conversion of Nitrosylhemoglobin to SNO-Hemoglobin

5        The hypothesis that the nitric oxide is transferred from the heme iron to a thiol residue, forming nitrosothiol upon oxygenation, was tested. Hemoglobin A (400  $\mu$ M) was deoxygenated by bubbling argon through a 1 ml solution in 100 mM phosphate (pH 7.4), for 45 minutes. Nitric oxide  
10        was added by injection of an appropriate volume of a 2 mM solution, stored under nitrogen, to achieve different NO/Hb ratios. The solutions were then exposed to air by vigorous vortexing in an open container. Samples were then analyzed by Saville assay and by chemiluminescence after UV  
15        photolysis. Data are shown as mean  $\pm$  standard error ( $n > 3$ ). Figure 13 shows that S-nitrosothiol is formed in this manner, and that the efficiency of this reaction is greatest at high ratios of heme to nitric oxide. Amounts are highest at very high NO/Hb ratios, i.e.,  $> 2:1$ . This  
20        result implies that nitrosyl Hb entering the lung is converted into SNO-Hb, as under physiological conditions the ratio of heme to NO is high.

Example 16: Effects Dependent upon Heme:NO Ratio

25        It was proposed that the binding of nitric oxide to the heme of the  $\beta$  chain was inherently unstable, and that the reason for lower yields of SNO-Hb at higher concentrations of nitric oxide, was a loss of bound nitric oxide as a result of this instability. Hemoglobin A (17.5  $\mu$ M) was deoxygenated by bubbling argon through a 1 ml  
30        solution in 100 mM phosphate (pH 7.4), for 45 minutes. Nitric oxide was added by sequential injections of an

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appropriate volume of a 2 mM solution, stored under nitrogen. Figure 14A: Difference spectra of the nitric oxide hemoglobin mixture and the starting deoxyhemoglobin spectrum are shown. Figure 14B: The peak wavelength of the difference spectra plotted against the concentration of nitric oxide added to the solution. These data show that addition of small amounts of nitric oxide (heme:NO ratios of approximately 70:1) produce predominantly nitrosylhemoglobin and some oxidized hemoglobin. However, nitric oxide additions of the order of 10  $\mu$ M result in the formation of oxidized hemoglobin. Heme:NO ratios at this point are approximately 7:1. As the concentration of nitric oxide is increased by further additions of nitric oxide, the predominant species formed becomes nitrosylhemoglobin (heme:NO ratio 1:1). The results in Figures 14A and 14B show that under anaerobic conditions, the addition of increasing quantities of nitric oxide to Hb results first in the production of nitrosylhemoglobin and then oxidized Hb (metHb). At very high levels of nitric oxide, nitrosyl-hemoglobin is once again seen as the nitric oxide first reduces metHb to deoxyHb (producing nitrite), then binds NO. This drives the conformational change of T-structure Hb to R-structure, stabilizing the  $\beta$  heme-nitric oxide bond. The appearance of oxidized Hb at heme to nitric oxide ratios of approximately 10:1 indicates the decay of the heme/NO bond to produce oxidized Hb and nitric oxide anion (nitroxyl). The presence of nitric oxide anion was confirmed by detection of  $N_2O$  in the gas phase by gas chromatography mass spectrometry and by the production of  $NH_2OH$ .

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Example 17: Effects upon Oxygenation of Nitrosyl-deoxyHb

Hemoglobin A (20.0  $\mu\text{M}$ ) was deoxygenated by bubbling argon through a 1 ml solution in 100 mM phosphate (pH 7.4), for 45 minutes. In both Figure 15A and Figure 15B, the lowest to the highest spectra indicate the sequential additions of air. These are difference spectra in which the pure deoxyHb spectrum occurs at zero absorbance. The peak at 419 nm is from nitrosylhemoglobin; oxidized hemoglobin absorbs at 405 nm.

- 10 In the experiments shown in Figure 15A, hemoglobin was gradually oxygenated by sequential additions of 10  $\mu\text{l}$  of room air by Hamilton syringe. Spectra are shown as difference spectra from the initial deoxyhemoglobin spectrum. In the experiments shown in Figure 15B, nitric
- 15 oxide (1  $\mu\text{M}$ ) was added by injection of 0.5  $\mu\text{l}$  of a 2 mM solution, stored under nitrogen. Final heme:NO ratio was 80:1. The solution was gradually oxygenated by sequential additions of 10  $\mu\text{l}$  of room air. Spectra are shown as difference spectra from the initial deoxyhemoglobin
- 20 spectrum. These data show the initial formation of a nitrosylhemoglobin peak, along with some formation of oxidized hemoglobin, which disappears after the addition of approximately 30  $\mu\text{l}$  of air. The results indicate that a small quantity of nitrosyl Hb is formed upon addition of
- 25 low ratios of nitric oxide to deoxy Hb, and that this nitrosyl Hb is lost upon oxygenation.

Example 18: Role of  $\beta 93\text{Cys}$  in Destabilizing Nitrosyl-Heme

- Recombinant hemoglobins were obtained from Clara Fonticelli at the University of Maryland School of
- 30 Medicine.  $\beta 93\text{Ala}$  represents a single amino acid substitution within human hemoglobin A, whilst  $\beta 93\text{Cys}$  represents a wild type control. Recombinant hemoglobin (5

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$\mu\text{M}$  containing either a wild type cysteine ( $\beta 93\text{Cys}$ ) or a mutant alanine ( $\beta 93\text{Ala}$ ) at position  $\beta 93$  was deoxygenated as in Figures 15A and 15B. Nitric oxide ( $1\ \mu\text{M}$ ) was added by injection of  $0.5\ \mu\text{l}$  of a  $2\ \text{mM}$  solution, stored under  
5 nitrogen. The final heme:NO ratio was 20:1. The solution was gradually oxygenated by sequential additions of  $10\ \mu\text{l}$  of room air. The absorption at  $418\ \text{nm}$  of difference spectra versus initial deoxyhemoglobin spectra is shown in Figure 16. These data indicate that within the mutant, a  
10 nitrosyl adduct was formed that was not lost upon addition of room air. However, the nitrosyl adduct formed within the wild type was lost after addition of greater than  $10\ \mu\text{l}$  of room air. This shows that NO is not lost from this nitrosyl ( $\text{FeII}$ ) heme in a mutant Hb that does not possess a  
15 thiol residue at position  $\beta 93$ . Therefore, this thiol, which is in close proximity to the heme within the R-structure, is critical for destabilizing the heme nitric oxide bond.

Example 19: SNO-Hb from Nitrosyl-Hb Driven by  $\text{O}_2$

20 Hemoglobin A ( $400\ \mu\text{M}$ ) was prepared in a  $1\ \text{ml}$  solution, in  $100\ \text{mM}$  phosphate ( $\text{pH } 7.4$ ). Nitric oxide was added by injection of an appropriate volume of a  $2\ \text{mM}$  solution, stored under nitrogen. The solutions were vortexed vigorously in an open container. Samples were then  
25 analyzed by Saville assay and by chemiluminescence after UV photolysis. The results in Figure 17 show that S-nitrosothiol Hb can be formed from oxyHb, but that the efficiency of this formation is critically dependent upon the ratio of heme to nitric oxide.

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Example 20: Formation of Oxidized Hb Dependent on Protein Concentration

Hemoglobin A was diluted to the concentrations indicated by the different symbols in Figure 18A and Figure 18B, in 50 ml of 100 mM phosphate buffer (pH 7.4). Nitric oxide was added by sequential injections of an appropriate volume of a 2 mM solution, stored under nitrogen. After each injection, the absorbance at 415 and 405 nm was measured. The ratio of these two absorbances was used to calculate the percentage content of oxidized hemoglobin (Figure 18A), and the absolute yield of oxidized hemoglobin (Figure 18B). ♦ represents 1.26  $\mu$ M hemoglobin, ■ represents 5.6  $\mu$ M hemoglobin, ▲ represents 7.0  $\mu$ M hemoglobin, X represents 10.3  $\mu$ M hemoglobin, ✕ represents 13.3  $\mu$ M hemoglobin, and • represents 18.3  $\mu$ M hemoglobin. These data show that only a small proportion of the nitric oxide added results in the formation of oxidized hemoglobin (<10%). Furthermore, this tendency to form oxidized hemoglobin is reduced at higher protein concentrations.

Example 21: Effect of Ionic Strength and NO:Hb Ratio on Extent of MetHb Formation

We proposed that the degree of hydrogen bonding between bound oxygen and the distal histidine was critical in determining the degree of oxidation of hemoglobin by nitric oxide. Therefore, we examined the degree of oxidation of hemoglobin by nitric oxide in a variety of buffers. 5 ml of phosphate buffer containing 300  $\mu$ M hemoglobin A (~95% oxyHb) was placed in a 15 ml vial. Nitric oxide was added from a stock solution, 2 mM, stored under nitrogen. Immediately after nitric oxide addition, the absorbance at 630 nm was measured, and the concentration of oxidized (metHb) was plotted, using 4.4 as

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the extinction coefficient for metHb at 630 nm. Experiments were performed in 1 M, 100 mM, and 10 mM sodium phosphate buffer (pH 7.4). The data in Figure 19 show higher oxidized hemoglobin formation in 1 M phosphate, which is indicative of a higher effective substrate concentration, as would be predicted by phosphate destabilization of the hydrogen bond between iron bound oxygen and the distal histidine. At the lowest concentrations of nitric oxide added, S-nitrosothiol was formed under all conditions (approximately 5  $\mu$ M). Additions of nitric oxide at concentrations of 30  $\mu$ M or greater resulted in the additional formation of nitrite. The presence of 200 mM borate within the buffer reduced oxidized hemoglobin and nitrite formation, whilst the presence of either 200 mM or chloride increased the formation of oxidized hemoglobin and nitrite. Addition of nitric oxide to hemoglobin in 10 mM phosphate buffer at a ratio of less than 1:30 (NO:Hemoglobin A) resulted in the formation of S-nitrosothiol without production of oxidized hemoglobin. S-nitrosothiol formation was optimized by adding the nitric oxide to hemoglobin in 10 mM phosphate, 200 mM borate, pH 7.4. Therefore, the balance between oxidation and nitrosothiol formation is dependent upon the ratio of nitric oxide to hemoglobin and the buffer environment.

Example 22: Oxygen-Dependent Vasoactivity of S-Nitrosohemoglobin: Contraction of Blood Vessels in R-Structure and Dilation in T-Structure

The details of this bioassay system have been published (Osborne, J.A., et al., J. Clin. Invest. 83:465-473 (1989)). In brief, New Zealand White female rabbits weighing 3-4 kg were anesthetized with sodium pentobarbital



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(30 mg/kg). Descending thoracic aorta were isolated, the vessels were cleaned of adherent tissue, and the endothelium was removed by gentle rubbing with a cotton-tipped applicator inserted into the lumen. The vessels  
5 were cut into 5-mm rings and mounted on stirrups connected to transducers (model TO3C, Grass Instruments, Quincy, MA) by which changes in isometric tension were recorded. Vessel rings were suspended in 7 ml of oxygenated Kreb's buffer (pH 7.5) at 37°C and sustained contractions were  
10 induced with 1  $\mu$ M norepinephrine.

Best attempts were made to achieve equivalent baseline tone across the range of oxygen concentrations; i.e., hypoxic vessels were contracted with excess phenylephrine. Oxygen tension was measured continuously using O<sub>2</sub>  
15 microelectrodes (Model 733 Mini; Diamond General Co., MI) (Young, W., *Stroke*, 11:552-564 (1980); Heiss, W.D. and Traupett, H., *Stroke*, 12:161-167 (1981); Dewhirst, M.W. et al., *Cancer Res.*, 54:3333-3336 (1994); Kerger, H. et al., *Am. J. Physiol.*, 268:H802-H810 (1995)). Less than 1% O<sub>2</sub>  
20 corresponds to 6-7 torr. Hypoxic vessels were contracted with excess phenylephrine to maintain tone. SNO-Hb[FeII]O<sub>2</sub> (SNO-oxyHb) preparations were synthesized and quantified as in Example 27; GSNO was prepared and assayed as described in Stamler, J.S. and Feelisch, M., "Preparation and  
25 Detection of S-Nitrosothiols," pp. 521-539 in *Methods In Nitric Oxide Research* (M. Feelisch and J.S. Stamler, eds.), John Wiley & Sons Ltd., 1996.

Hemoglobin is mainly in the R (oxy)-structure in both 95% O<sub>2</sub> or 21% O<sub>2</sub> (room air) (M.F. Perutz, pp. 127-178 in  
30 *Molecular Basis of Blood Diseases*, G. Stamatayanopoulos, Ed. (W.B. Saunders Co., Philadelphia, 1987); Voet, D. and Voet, J.G. (John Wiley & Sons Inc., New York, 1995) pp. 215-235). Hb and SNO-Hb both contract blood vessels over

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this range of O<sub>2</sub> concentrations. That is, their hemes sequester NO from the endothelium. The functional effects of these hemoproteins in bioassays are not readily distinguished (Figure 20A). Concentration-effect responses of SNO-Hb are virtually identical to those of native Hb in 95% O<sub>2</sub>--i.e., in R-structure (curves are not different by ANOVA; n=12 for each data point). Comparable contractile effects were seen with up to 50  $\mu$ M SNO-oxyHb/oxyHb--i.e., at doses where the responses had plateaued. Similar concentration-effect responses were observed in 21% O<sub>2</sub>, under which condition Hb/SNO-Hb is ~99% saturated.

On the other hand, hypoxia (<1% O<sub>2</sub> [-6 mm Hg] simulating tissue PO<sub>2</sub>) which promotes the T-structure (M.F. Perutz, pp. 127-178 in *Molecular Basis of Blood Diseases*, G. Stamatayanopoulos, Ed. (W.B. Saunders Co., Philadelphia, 1987); Voet, D. and Voet, J.G. (John Wiley & Sons Inc., New York, 1995) pp. 215-235), differentiates Hb and SNO-Hb activities: Hb strongly contracts blood vessels in T structure whereas SNO-Hb does not (Figure 20B). Concentration-effect responses of SNO-Hb and Hb are significantly different <1% O<sub>2</sub> (~6 torr); i.e. in T-structure. Native deoxyHb is a powerful contractile agent whereas SNO-deoxyHb has a modest effect on baseline tone. (In most experiments SNO-Hb caused a small degree of contraction at lower doses and initiated relaxations at the highest dose; in some experiments (see Figure 21C) it caused dose-dependent relaxations.) n=13 for each data point; \*P<0.05; \*\*\*P<0.001 by ANOVA.

SNO-Hb relaxations are enhanced by glutathione through formation of S-nitrosoglutathione (GSNO) (Figure 20C). The potentiation of SNO-Hb vasorelaxation by glutathione is inversely related to the PO<sub>2</sub> (Figure 20C) because NO group transfer from SNO-Hb is promoted in the T-structure.

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- Specifically, transnitrosation of glutathione by SNO-Hb--forming the vasodilator GSNO--is accelerated in T-structure (<1% O<sub>2</sub>). Addition of 10  $\mu$ M glutathione to bioassay chambers potentiates the vasorelaxant response of SNO-Hb.
- 5 The potentiation is greatest under hypoxic conditions; i.e., the curve for <1% O<sub>2</sub> shows a statistically significant difference from both the 95% and 21% O<sub>2</sub> curves (P<0.001), which are not different from one another by ANOVA (n=6 for all data points). High concentrations of
- 10 glutathione (100  $\mu$ M-1 mM) further potentiate SNO-Hb relaxations, such that the response is virtually identical to that seen in the presence of GSNO in Figure 20D. Glutathione at 10  $\mu$ M has no effect on native Hb contractions.
- 15 In contrast, the vasorelaxant effects of S-nitrosoglutathione are largely independent of PO<sub>2</sub> (Figure 20D) and unmodified by superoxide dismutase. (Data not shown.) Concentration-effect responses of S-nitrosoglutathione (GSNO) are largely independent of PO<sub>2</sub> in
- 20 the physiological range (n=6 at each data point). Results are consistent with known resistance of GSNO to O<sub>2</sub>/O<sub>2</sub><sup>-</sup> inactivation (Gaston, B. et al., *Proc. Natl. Acad. Sci. USA*, 90:10957-10961 (1993)). Thus, in T-structure, relaxation by SNO overwhelms the contraction caused by NO
- 25 scavenging at the heme, whereas the opposite is true in R-structure.

Example 23: Bioactivity of Intraerythrocytic S-Nitrosohemoglobin (SNO-RBCs)

- Contractile effects of red blood cells are reversed by
- 30 intracellular SNO-Hb in low but not high PO<sub>2</sub>--i.e., under conditions that promote the T-structure. Low and high dose

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effects of SNO-RBCs are shown in Figures 21A and 21B, respectively.

Preparation of vessel rings and methods of bioassay are described in Example 22. SNO-oxyHb was synthesized and quantified as described in Example 27. Red blood cells containing SNO-Hb (SNO-RBCs) were synthesized by treatment with tenfold excess S-nitrosocysteine over hemoglobin for 5-10 min. Under this condition, red blood cells are bright red and contain SNO-oxyHb; methHb was not detectable in these experiments.

Red blood cells containing SNO-Hb (SNO-RBCs) function in vessel ring bioassays like cell-free SNO-Hb. In particular, low concentrations of SNO-RBCs ( $\sim 0.1 \mu\text{M}$  SNO-Hb) elicited modest contractile effects in 95%  $\text{O}_2$ , but not under hypoxia (Figure 21A). In 95%  $\text{O}_2$ , both SNO-RBCs ( $\sim 0.1 \mu\text{M}$  SNO-Hb[FeII] $\text{O}_2$ ) and native RBCs produced modest contractile effects that were not readily distinguished. The contractions by RBCs tended to be greater under hypoxic conditions ( $< 1\%$   $\text{O}_2$ ), whereas those of SNO-RBCs were reversed (slight relaxant effects were seen). These  $\text{O}_2$ -dependent responses of SNO-RBCs closely resemble those of cell-free preparations. Hemolysis was minor and could not account for the observed effects.

At higher concentrations, SNO-RBCs produced small transient relaxations in 95%  $\text{O}_2$  and larger sustained relaxations under hypoxia (Figure 21B), much like cell-free SNO-Hb in the presence of glutathione. For example, SNO-RBCs ( $\sim 1 \mu\text{M}$  SNO-Hb[FeII] $\text{O}_2$ ) caused  $32.5 \pm 1.2\%$  relaxation that lasted  $14.5 \pm 0.7$  min. in 95%  $\text{O}_2$  versus  $61 \pm 10\%$  relaxation that lasted  $23 \pm$  min. in  $< 1\%$   $\text{O}_2$  ( $n=3-4$ ;  $P<0.05$ ). In contrast, red blood cells containing no SNO-Hb produced small contractions (less than those of cell-free Hb) that are potentiated by hypoxia ( $13 \pm 2.0\%$  in 95%  $\text{O}_2$  vs.  $25 \pm 5\%$  in

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<1% O<sub>2</sub>; P<0.05). Hemolysis in these experiments was minor and could not account for the extent of relaxation by SNO-RBCs.

In 95% O<sub>2</sub>, SNO-RBCs (~1 μM SNO-Hb[FeII]O<sub>2</sub>) produced relaxations of aortic rings, whereas native RBCs produced slight contractions. Both effects were more prominent at low PO<sub>2</sub>. That is, relaxations and contractions of intraerythrocytic SNO-Hb and Hb, respectively, were greater and longer-lived in <1% O<sub>2</sub> than in 95% O<sub>2</sub>. The O<sub>2</sub>-dependent responses of SNO-RBCs mimicked those of cell-free SNO-Hb in the presence of glutathione. Hemolysis in these experiments was minor and could not account for the extent of relaxation by SNO-RBCs.

The normal response of systemic arteries to hypoxia is dilation, and to high PO<sub>2</sub>, contraction. The responses of vessel rings to changes in PO<sub>2</sub> in the presence of SNO-Hb and Hb were tested (Figure 21C). Vessel rings were contracted with phenylephrine under hypoxic conditions (6-7 torr) and then exposed to either 1 μM Hb or SNO-Hb. Hb produced progressive increases in vessel tone, while SNO-Hb caused relaxations. Introduction of 95% O<sub>2</sub> led to rapid contractions in both cases. Thus, structural changes in SNO-Hb effected by PO<sub>2</sub> are rapidly translated into contractions or relaxations, whereas Hb contracts vessels in both R- and T-structures. Thus, Hb opposes the physiological response and SNO-Hb promotes it (Figure 21C). Direct effects of O<sub>2</sub> on smooth muscle operate in concert with SNO-Hb to regulate vessel tone.

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Example 24: Influence of O<sub>2</sub> Tension on Endogenous Levels of S-Nitrosohemoglobin (SNO/Hb) and Nitrosyl Hemoglobin (FeNO/Hb)

Allosteric control of SNO-Hb by O<sub>2</sub> was assessed in vivo by perturbation of the periarteriolar oxygen gradient. In animals breathing room air (21% O<sub>2</sub>), the precapillary resistance vessels (100-10  $\mu$ m) are exposed to PO<sub>2</sub>s as low as 10-20 torr (Duling, B. and Berne, R.M. *Circulation Research*, 27:669 (1970); Popel, A.S., et al., (erratum *Am. J. Physiol.* 26(3) pt. 2) *Am. J. Physiol.* 256, H921 (1989); Swain, D.P. and Pittman, R.N., *Am. J. Physiol.* 256, H247-H255 (1989); Buerk, D. et al., *Microvasc. Res.*, 45:134-148 (1993)) (confirmed here) which promotes the T-structure in Hb. Raising the inspired oxygen concentration to 100% translates to periarteriolar PO<sub>2</sub>s only as high as 40 mm Hg (Duling, B. and Berne, R.M. *Circulation Research*, 27:669 (1970); Popel, A.S., et al., (erratum *Am. J. Physiol.* 26(3) pt. 2). *Am. J. Physiol.* 256, H921 (1989); Swain, D.P. and Pittman, R.N. *Am. J. Physiol.* 256, H247-H255 (1989); Buerk, D. et al., *Microvasc. Res.*, 45:134-148 (1993)); i.e., breathing 100% O<sub>2</sub> may not fully maintain the R-structure in Hb in the microcirculation. Elimination of the periarteriolar O<sub>2</sub> gradient (artery-arteriole and arterial-venous difference in PO<sub>2</sub>) is accomplished in hyperbaric chambers by applying 3 atmospheres of absolute pressure (ATA) while breathing 100% O<sub>2</sub> (Tibbles, P.M. and Edelsberg, J.S., *N.E.J.M.*, 334:1642-1648 (1996)).

Adult male Sprague-Dawley rats (290-350 g) were anesthetized with sodium pentobarbital (50 mg/kg IP), intubated and ventilated with a small animal respirator (Edco Scientific Inc., Chapel Hill, NC) at a rate and tidal volume to maintain normal values of PaCO<sub>2</sub> (35-45 mm Hg; PaCO<sub>2</sub> = systemic arterial blood carbon dioxide tension).

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The femoral vein and artery were cannulated for infusion of drugs and for continuous monitoring of systemic blood pressure, respectively. Aliquots of arterial blood (200  $\mu$ l) were drawn periodically to measure blood gas tensions and pH (Instrumentation Laboratory Co., model 1304 blood gas/pH analyzer). The blood was replaced intravenously with three volumes of normal saline. The inspired  $O_2$  concentration was varied using premixed gases balanced with nitrogen. The tissue  $PO_2$  was measured continuously with polarographic platinum microelectrodes (50  $\mu$ m O.D. coated with hydrophobic gas permeable Nafion) implanted stereotaxically in both the right and left hippocampus (AP-3.4 mm, ML+2.2 mm), caudate putamen nucleus and substantia nigra (see coordinates below) (Young, W., *Stroke*, 11:552-564 (1980); Heiss, W.D. and Traupett, H., *Stroke*, 12:161-167 (1981); Dewhirst, M.W. et al., *Cancer Res.*, 54:3333-3336 (1994); Kerger, H. et al., *Am. J. Physiol.*, 268:H802-H810 (1995)). The  $PO_2$  electrodes were polarized to -0.65V against a distant Ag/AgCl reference located on the tail and the current flow was measured using a low-impedance nA-meter. Regional arterial  $PO_2$  was adjusted by changing the inspired  $O_2$  concentration and atmospheric pressure.

Polarographic hydrogen ( $H_2$ )-sensitive microelectrodes were implanted stereotaxically in the substantia nigra (AP -5.3 mm, ML -2.4 mm to the bregma, depth 3.2 mm), caudate putamen nucleus (CPN) (AP +0.8 mm, ML -2.5 mm, depth 5.2 mm) and parietal cortex, for measurement of regional blood flow (Young, W., *Stroke*, 11:552-564 (1980); Heiss, W.D. and Traupett, H., *Stroke*, 12:161-167 (1981)). The microelectrodes were made from platinum wire and insulated with epoxy, with the exception of the tip (1 mm) which was coated with Nafion. For placement, the electrodes were mounted on a micromanipulator and the rat's head was

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immobilized in a Kopf stereotaxic frame. H<sub>2</sub>-sensitive electrodes were polarized to +400 mV against a distant reference electrode on the tail, and the polarographic current was measured using a low-impedance nA meter during  
5 and after the inhalation of hydrogen gas (2.5%) for 1 min. Both the hydrogen clearance curves and voltage for oxygen measurements were made using PC WINDAQ (software, DI-200 AC, Dataq Instruments, Inc., Akron, OH). Cerebral blood flow was calculated using the initial slope method (Young,  
10 W., *Stroke*, 11:552-564 (1980); Heiss, W.D. and Traupett, H., *Stroke*, 12:161-167 (1981)). Regional blood flow responses were monitored for 30 min. prior to and 30 min. following drug administration; hemoglobins were given at time 0.

15 Blood was drawn from indwelling catheters in the carotid artery (arterial blood that perfuses the brain) and superior vena cava/right atrium (venous return to the heart) of 5 rats exposed first to room air (21% O<sub>2</sub>) and then 100% O<sub>2</sub> + 3 ATA in a hyperbaric chamber. Levels of  
20 SNO-Hb and nitrosyl Hb (Hb[Fe]NO) were determined from these samples as a measure of SNO-Hb and nitrosyl Hb (Hb[Fe]NO; FeNO/Hb in Figure 9) in blood that perfuses the brain.

Blood samples were transported on ice for immediate  
25 processing and analysis. After centrifugation at 800 g for 10 min, the packed red blood cells were isolated, washed with a 2-fold volume excess of PBS, pH 7.4, resuspended, and the PBS removed after a further centrifugation. Hemolysis was then accomplished by incubation for 10 min  
30 with 4-fold excess deionized water containing 0.5 mM EDTA, followed by purification of hemoglobin by rapidly desalting over a G-25 Sephadex chromatography spin column (10 to 30-fold volume excess) in PBS at room temperature. Total Hb



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concentration was determined by the visible spectrophotometric method. Hb species present were converted to Hb(FeII)NO (by addition of dithionite in the presence of excess S-nitrosocysteine), which was then  
5 measured using the millimolar extinction coefficient of 135.4 at 418 nm.

Each sample of hemoglobin was diluted to 200  $\mu$ M and paired aliquots were treated with an equal volume of either distilled water or 7.5-fold molar excess HgCl<sub>2</sub> (which  
10 selectively cleaves thiol-bound NO). Higher concentrations of HgCl<sub>2</sub> cause Hb to precipitate (A. F. Riggs, R. A. Wolbach, *J. Gen. Physiol.* 39:585, 1956). The HgCl<sub>2</sub> concentration can be reduced to 4-fold excess over protein with quite comparable results. A 6-fold excess HgCl<sub>2</sub>  
15 concentration and an incubation time of 1-10 minutes has been used also (determined empirically for each reaction mixture). Organic mercurials have been used instead of hemoglobin. They do not cause precipitation, even at high concentrations, but they react more slowly. In all cases,  
20 the mercurial selectively cleaves the NO group from thiols and preserves binding at the heme. NO was measured by the photolysis-chemiluminescence method, in which NO is photolytically liberated from thiols (SNO-Hb) or hemes (Hb[FeII]NO) and the chemiluminescent product of its  
25 reaction with ozone is measured. Standard curves were generated using S-nitrosoglutathione. See "Assay for S-Nitrosohemoglobin and Nitrosyl(FeII)-Hemoglobin" in *Materials and Methods for Assays* section at beginning of Exemplification and Methods section for Example 8.

30 The mean O<sub>2</sub> saturation of venous blood (room air) was 69%; of arterial blood (room air) was 93%; of venous blood (100% + 3 ATA) was also 93% and of arterial blood (100% + 3 ATA) was 100% (Figure 22). Numerous statistical

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comparisons were highly significant. For example, SNO-Hb venous 100% O<sub>2</sub> + 3 ATA vs. SNO-Hb venous 21% O<sub>2</sub>, P=0.004; and nitrosyl Hb venous 21% O<sub>2</sub> vs. arterial 21% O<sub>2</sub> P=0.008. On the other hand, SNO-Hb and nitrosyl Hb were not

5 different in artery 21% O<sub>2</sub> compared with venous 100% + 3 ATA (which have identical O<sub>2</sub> saturations), nor did the differences reach significance between venous and arterial 100% O<sub>2</sub> + 3 ATA. n=5 for all measurements.

In 21% O<sub>2</sub>, venous blood contained mostly nitrosyl Hb, 10 whereas arterial blood contained significant amounts of SNO-Hb (Figure 22). On the other hand, SNO-Hb predominated in both arterial and venous blood in 100% O<sub>2</sub> + 3 ATA (Figure 22). In hyperbaric conditions, the tissues are oxygenated primarily by O<sub>2</sub> dissolved in plasma. 15 Physiologically circumventing the unloading of O<sub>2</sub> by Hb alters the endogenous SNO/nitrosyl Hb balance. The data show that SNO-Hb appears to form endogenously in R-structure whereas SNO is released in the T-structure (compare venous 21% O<sub>2</sub> (T-state) with arterial 100% O<sub>2</sub> + 3 20 ATA (R-state)).

This structure-function relationship *in vivo* is consistent with both the *in vitro* pharmacology and the molecular model suggesting that 1) O<sub>2</sub> is an allosteric effector of Hb S-nitrosylation; 2) binding of NO to hemes 25 of Hb is favored in the T-structure; (some of the NO released during arterial-venous (A-V) transit appears to be autocaptured at the hemes) and 3) maintaining endogenous SNO-Hb in the R-structure by eliminating the A-V O<sub>2</sub> gradient preserves levels of SNO (compare venous 100% O<sub>2</sub> + 30 3 ATA with arterial 21% O<sub>2</sub>). Thus, it can be predicted that SNO-Hb should improve cerebral blood flow in 21% O<sub>2</sub>, under which condition SNO is readily released during A-V

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transit, but not under the hyperoxic conditions that maintain the R-structure in artery and vein.

Example 25: O<sub>2</sub>-Dependent Effects of SNO-Hb and Hb on Local Cerebral Blood Flow

- 5       The cerebrovascular effects of SNO-Hb were measured in adult male Sprague-Dawley rats using O<sub>2</sub> and H<sub>2</sub> (blood flow)-sensitive microelectrodes that were placed stereotaxically in several regions of the brain as for Example 24.
- 10       SNO-Hb increases blood flow under tissue hypoxia, whereas it decreases blood flow under hyperoxia. In contrast, Hb decreases blood flow irrespective of the PO<sub>2</sub>. Comparative effects of SNO-Hb (●) and Hb (■) (1 μmol/kg infused over 3 minutes) on local blood flow in substantia
- 15   nigra (SN), caudate putamen nucleus, and parietal cortex are shown for three different conditions. In 21% O<sub>2</sub>, SNO-Hb improved blood flow in all three regions of the brain tested, whereas native Hb decreased local blood flow, paradoxically attenuating O<sub>2</sub> delivery to hypoxic tissues
- 20   (Figures 23A, 23B and 23C; all curves are highly statistically significantly different from one another and from baseline by ANOVA). In rats breathing 100% O<sub>2</sub>, where the periarteriolar O<sub>2</sub> gradient has been essentially eliminated, the increase in flow to SNO-Hb was
- 25   significantly attenuated (i.e., only the SN increase reached statistical significance), but the Hb-mediated decrease in flow was preserved (Figures 23D, 23E and 23F; all curves remain different from one another by ANOVA to P<0.05). In 100% O<sub>2</sub> + 3 ATA, both SNO-Hb and Hb tended to
- 30   decrease cerebral flow to similar extents (Figures 23G, 23H and 23I; curves are not different by ANOVA). S-nitrosoglutathione (GSNO) increased brain perfusion in 100%

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O<sub>2</sub> and 100% O<sub>2</sub> + 3 ATA, reversing protective vasoconstriction. Baseline blood flow was decreased by ~10% under 100% O<sub>2</sub> + 3 ATA as compared to 100% O<sub>2</sub>. n=7 for all data points. Values of tissue/microvascular PO<sub>2</sub> ranged  
5 from 19-37 mm Hg in 21% O<sub>2</sub>; from 68-138 mm Hg in 100% O<sub>2</sub>; and from 365-538 mm Hg in 100% + 3 ATA (Duke University Medical Center Hyperbaric Chambers).

The effects of Hb and SNO-Hb on local blood flow in the parietal cortex can be seen by comparing the results  
10 shown in Figures 23C, 23I and 23F. In 21% O<sub>2</sub> (tissue/microvascular PO<sub>2</sub> 19 to 37 mm Hg), Hb reduced blood flow whereas SNO-Hb augmented blood flow (Figure 23C). The increase in blood flow in response to SNO-Hb was significantly attenuated in 100% O<sub>2</sub> (Figure 23F; tissue PO<sub>2</sub>  
15 68 to 138 mm Hg) and was converted to decreases in flow in 100% O<sub>2</sub> plus 3 ATA (Figure 23I; tissue PO<sub>2</sub> 365 to 538 mm Hg). In contrast, responses to Hb or GSNO were not oxygen-dependent: Hb decreased blood flow while GSNO increased it, irrespective of PO<sub>2</sub>. Thus, SNO-Hb uniquely regulates  
20 blood flow in response to the physiological oxygen gradient in resistance arterioles.

SNO-Hb acts like native Hb (net NO scavenger) when it is in the R (oxy)-structure and like GSNO (net NO donor) in the T (deoxy)-structure. The results are consistent with  
25 the conclusion that SNO-Hb is a nitrosothiol whose vasoactivity is allosterically controlled by PO<sub>2</sub>.

Example 26: Hemodynamics of Cell Free and Intraerythrocytic SNO-Hb, Hb and GSNO at Different O<sub>2</sub> Concentrations

30 Rats were anesthetized by intraperitoneal injection of pentobarbital, and the femoral arteries and veins accessed by local cutdown. The artery was then cannulated and the

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blood pressure monitored continuously using a P23 XL pressure transducer (Viggo Spectramed, Oxnard, CA) attached to a Gould recorder. The femoral vein was used for infusion of drugs and red blood cells containing SNO-Hb (1 ml over 1 min.) and an IBM PC (WINDAQ 200, Dataq Instruments, Inc.; Akron, OH) was used for data acquisition.

Drugs were infused through the femoral vein at 1  $\mu$ mol/kg infused over 1 minute after blood pressure had stabilized (approximately 30 min). Measurements shown (Figure 24A) were taken at 10 min. post-infusion of drug. Similar responses were seen at 3 and 20 min. SNO-Hb produced significantly less of an increase in blood pressure than Hb ( $P < 0.05$ ), whereas GSNO decreased blood pressure.  $P < 0.05$  vs. SNO-Hb;  $*P < 0.05$ ,  $**P < 0.01$ ) vs. baseline blood pressure.  $n = 5-6$  for each drug.

Infusions of SNO-RBCs also lowered blood pressure consistent with a GSNO-like effect (Figure 24B). SNO-RBCs produced dose-dependent hypotensive effects (similar to those of cell-free SNO-Hb) ( $P < 0.001$  at all points vs. baseline). The hypotensive effects of SNO-RBCs were potentiated by pre-administration of the NO synthase inhibitor  $N^G$ -monomethyl-L-arginine (L-NMMA; 50 mg/kg).  $n = 8$  for each data point. Curves different by ANOVA ( $P < 0.01$ ),  $*P < 0.05$  vs. L-NMMA. The amount of hemolysis in these experiments was trivial. Infusion of the hemolysate had no effect on blood pressure.

NO synthase inhibition increases tissue  $O_2$  consumption by relieving the inhibition of mitochondrial respiration produced by NO in the tissues (King, C.E. et al., *J. Appl. Physiol.*, 76(3):1166-1171 (1994); Shen, W. et al., *Circulation*, 92:3505-3512 (1995); Kobzik, L. et al., *Biochem. Biophys. Res. Comm.*, 211(2):375-381 (1995)). This

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should, in turn, increase the periarteriolar  $O_2$  gradient which might explain some of the potentiation. However, other factors, such as a change in tone or distribution of blood flow imposed by L-NMMA, may well contribute. The effects of SNO-Hb on blood pressure are consistent with SNO being released in resistance arterioles to compensate for NO scavenging at the heme iron.

Example 27: Synthesis of S-Nitrosohemoglobins

Materials and Methods

10 L-cysteine hydrochloride, glutathione, sulfanilamide, and N-(1-naphthyl)ethylenediamine (NED) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium nitrate and potassium ferricyanide were purchased from Aldrich Chemical Co. (Fairlawn, NJ). G-25 Sephadex (fine) was purchased from Pharmacia Biotech (Uppsala, Sweden). Purified (~99.00%) human HbA<sub>0</sub> was prepared as previously described, and was stored at  $-80^\circ\text{C}$  (R. G. Kilbourn, G. Joly, B. Cashon, J. DeAngelo, J. Bonaventura. *Biochem. Biophys. Res. Comm.* 199:156, 1994). The final buffer was lactated Ringer's solution, pH 7.4. Nitric oxide solutions were prepared in tonometers by bubbling high-purity, KOH-scrubbed NO gas through rigorously degassed solutions of phosphate-buffered saline (PBS) or deionized water. EDTA is ethylenediaminetetraacetic acid.

25 Synthesis of S-Nitroso-Oxyhemoglobin (SNO-Hb[Fe(II)] $O_2$ )

The principal technical challenges in synthesis of S-nitrosooxyhemoglobin (SNO-Hb[Fe(II)] $O_2$ ) are to selectively nitrosylate specific thiols and to avoid oxidation of the heme. The method of preparation of SNO-Hb(Fe(II)] $O_2$  is thus quite different from that previously described for the synthesis of other S-nitrosoproteins (J. S. Stamler, D. I.

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Simon, J. A. Osborne, M. E. Mullins, O. Jaraki, T. Michel, D. J. Singel, J. Loscalzo. *Proc. Natl. Acad. Sci.* 89:444, 1992). The rate of S-nitrosylation is accelerated, while the rate of oxidation of the hemes is slowed, in alkaline  
5 buffer.

Hemoglobin (Hb)A<sub>0</sub> was purified from human red blood cells as previously described (Kilbourn, R.G. et al., *Biochem. Biophys. Res. Commun.*, 199:155-162 (1994)). HbA<sub>0</sub> (0.5-1.0 mM) was dialyzed against 2% aerated borate, 0.5 mM  
10 EDTA (pH 9.2) at 4°C for 12-16 hours. The oxyHb concentration was determined based on the optical absorbance at 577 nm (i.e., using the millimolar extinction coefficient 14.6). An excess of nitrosylating agent is used for effective synthesis, but steps must be taken to  
15 ensure the selective modification of Cysβ93 (over other thiols and hemes in Hb).

Hb was reacted with 10-fold molar excess S-nitrosocysteine (CysNO) which was synthesized immediately before use in high concentration by a modification of  
20 standard procedure (see, for example, Stamler, J.S. and Feelisch, M., "Preparation and Detection of S-Nitrosothiols," pp. 521-539 in *Methods In Nitric Oxide Research*, M. Feelisch and J.S. Stamler, eds., John Wiley & Sons Ltd., 1996) as follows. L-cysteine hydrochloride (1.1  
25 M) dissolved in 0.5 N HCl/0.5 mM EDTA was reacted with an equal volume of 1 M NaNO<sub>2</sub> (sodium nitrite) dissolved in water, to form CysNO (the ratio of cysteine to nitrite influences the SNO-Hb product and activity profile), and is neutralized prior to addition to the hemoglobin solution by  
30 dilution in 100-200 mM PBS (pH 7.4 to 8.0, with 0.5 mM EDTA). The concentration of CysNO was then adjusted by dilution in PBS, pH 8.0, to yield a working CysNO solution (pH 6-7). Oxyhemoglobin (>100 μM in borate, pH 9.1-9.2)

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was S-nitrosylated by incubation with a 10-fold molar excess of CysNO over Hb (ratio influences product critically). Periods of incubation are determined by the desired synthetic preparation; i.e., a desired ratio of SNO/tetramer, desired met- to oxy- to nitrosyl-Hb ratios, polynitrosated or non-polynitrosated. For example, 10 min. is a preferred time for SNO-oxyHb with 2 SNO per tetramer. The reaction was stopped by rapid transfer of the reaction mixture to a column of fine Sephadex G-25 (bed volume should be 10 to 30-fold excess over that of the reaction mixture) preequilibrated with 100 mM PBS pH 7.4, 0.5 mM EDTA. Typically, a 150  $\mu$ l sample of the mixture was added to a 4.5 ml column measuring 12 mm (inner diameter). The column was then centrifuged at 800-1200 g for 60 seconds and the effluent containing purified SNO-oxyHb collected in a 1.5 ml airtight plastic vial that was subsequently kept on ice and protected from light.

Total Hb concentration was determined by the sequential addition of dithionite and excess CysNO, which results in the conversion of the Hb species present to Hb(FeII)NO. The millimolar extinction coefficient of Hb(FeII)NO is 135.4 (based on heme) at 418 nm. SNO-Hb(FeII)O<sub>2</sub> is quite stable, although it is best that samples be made fresh daily.

The S-nitrosothiol content of SNO-Hb was determined by a modification of the method of Saville. By this method, Hg<sup>++</sup> (derived from HgCl<sub>2</sub> or from organic mercurials such as 4-(chloromercuri)benzenesulfonate; 5 to 10-fold excess over Hb)-displaced NO equivalent was assayed by the diazotization of sulfanilamide and the subsequent coupling with the chromophore N-(1-naphthyl)ethylenediamine (NED). From measurements of the optical density at 540 nm, SNO concentrations were determined against those of the



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standard, S-nitrosoglutathione (GSNO). These assays were carried out in a 96-well microplate reader (Molecular Devices Corp. Sunnyvale, CA). Each sample well contained 5  $\mu$ l SNO-Hb/95  $\mu$ l 0.5 N HCl/100  $\mu$ l sulfanilamide/100  $\mu$ l NED.

- 5 Triton X-100 (0.03-0.1%) has been used if necessary to prevent precipitation of hemoglobin.

- Incubations of CysNO with HbA resulted in different synthetic products (and different activities) over time. For example, with 10 min incubations, the Hb preparation  
10 contained  $1.857 \pm 0.058$  SNO groups per tetramer, and is approximately 12-15% metHb and 1-3% nitrosyl(FeII)-hemoglobin. Capillary electrophoretic analysis revealed a mixture of three protein peaks. MetHb was then reduced (lowered from 13% to 2% with 100-fold excess NaCNBH<sub>3</sub>,  
15 dissolved in PBS, pH 8.0) under anaerobic conditions (achieved by purging with argon gas for a minimum of 10 minutes). Lower concentrations of NaCNBH<sub>3</sub> or treatment of the samples under aerobic conditions were not effective in lowering the metHb concentration, and alternative measures  
20 to reduce the heme resulted in SNO reduction. The resulting mixture was rapidly added to a column of fine Sephadex G-25 (20 to 30-fold volume excess) preequilibrated with 100 mM PBS pH 7.4, 0.5 mM EDTA. The final S-nitrosothiol/Hb tetramer ratios were not significantly  
25 different from those measured in samples degassed and treated with PBS only: losses in SNO/Hb ratio relative to the starting ratios were consistent with the expected time-dependent decay of SNO-deoxyHb and could be reduced to an insignificant loss by taking preparation time into  
30 consideration. NaCNBH<sub>3</sub> treatment of a sample with a mean SNO/Hb ratio of ~1 decreased the metHb content from 5.6% to 0.63%.

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Variations in Methods of Synthesis

Nitrosyl(FeII)-hemoglobin and methHb contamination of SNO-oxyHb preparations or compositions of less than about 2% are acceptable, inasmuch as they do not seem to alter bioactivity of SNO-oxyHb, and enable O<sub>2</sub> binding measurements, that is, P<sub>50</sub> determinations. Bioactivity can be modified and varied by controlling the proportion of SNO-methHb (high and low spin) and nitrosyl(FeII)-hemoglobin in the composition. The spin state of methHb can be controlled by the heme ligand: cyan-methHb is low spin and aquo-met Hb (H<sub>2</sub>O bound as ligand) is high spin. The desired proportion of nitrosylhemoglobin can be controlled (see, inter alia, Example 16). High yield SNO-methHb, SNO-nitrosyl(FeII)-hemoglobin can be formed by using the heme-liganded protein as starting material. Carbomonoxy Hb can be made by gassing with CO under anaerobic conditions. HbCO can then be used as starting material to make SNO-carboxyl-Hb. Various combinations of Hb[FeNO][FeCO] can also be used as starting material.

20 Synthesis of S-Nitroso-Deoxyhemoglobin (SNO-Hb[Fe(II)])

Isolated SNO-deoxyHb is synthesized in an anaerobic environment (glove box) using the general approach described for SNO-oxyHb. The hemoglobin solution and other materials for synthesis are allowed to equilibrate overnight in the glove box. The UV-visible spectrum of the Hb solution should be that of pure deoxyHb (Soret region peak at 430 nm) before synthesis is initiated. Purified SNO-deoxyHb samples are transferred to tonometers or sealed cuvettes before removal from the glove box. Isolated SNO-deoxyHb is highly unstable and must be used immediately.

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Synthesis of S-nitroso-Methemoglobin (Abbreviated as SNO-MetHb or SNO-Hb[Fe(III)])

Isolated methemoglobin was produced by reacting oxyhemoglobin (0.5-1.0 mM, pH 7.4 in 150 mM phosphate buffer solution, with 0.5 mM EDTA) with a 10-fold molar excess (over hemoglobin tetramer) of either  $\text{NaNO}_2$  or potassium ferricyanide  $\text{K}_3\text{Fe}(\text{CN})_6$  at room temperature for 10 min. The reaction mixture was desalted across a column of fine G-25 Sephadex (10-fold volume excess, preequilibrated with PBS, pH 7.4, with 0.5 mM EDTA) by centrifugation. The completeness of conversion to methemoglobin was then confirmed spectrophotometrically. (V. G. Kharitonov, J. Bonaventura, V. S. Sharma. Interactions of nitric oxide with heme proteins using UV-vis spectroscopy. In: *Methods in Nitric Oxide Research*, M. Feelisch and J. S. Stamler, eds., 1996. John Wiley and Sons Ltd., Chichester, England.) Methemoglobin was S-nitrosylated by incubation with CysNO, with the duration determined by the desired extent of S-nitrosylation, as described in the synthesis of SNO-Hb(FeII)O<sub>2</sub>. The reaction was stopped by rapid transfer of the reaction mixture to a G-25 Sephadex chromatography column (10 to 30-fold volume excess over reaction mixture), followed by rapid centrifugation and collection of the effluent in a plastic vial. Purified SNO-metHb is inherently unstable and should be resynthesized at frequent intervals.

Measurement of NO/SNO Content of Partially Nitrosylated DeoxyHb and S-Nitroso-Oxyhemoglobin in Reaction Mixtures Under Physiological Conditions

Isolated SNO-Hb(FeII)O<sub>2</sub> and Hb(FeII)NO were prepared at relatively physiological ratios of NO to Hb. SNO-Hb(FeII)O<sub>2</sub> was synthesized as described above and diluted

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to 1  $\mu\text{M}$  in 100  $\mu\text{M}$   $\text{Hb(FeII)O}_2$ /PBS pH 7.4/0.5 mM EDTA, resulting in a 1:100 ratio of  $\text{SNO-Hb(FeII)O}_2$  to  $\text{Hb(FeII)O}_2$ .  $\text{Hb(FeII)NO}$  (1  $\mu\text{M}$  NO:100  $\mu\text{M}$  deoxy Hb) was prepared by the addition of saturated NO solution to 100  $\mu\text{M}$  (final)

- 5 deoxyHb/PBS pH 7.4/0.5 mM EDTA under anaerobic conditions. NO was measured by the photolysis-chemiluminescence method in the absence (NO bound) or presence (NO post Hg) of  $\text{HgCl}_2$  (final 600  $\mu\text{M}$ ). Data (n=5) in Figure 25 represent mean  $\pm$  SEM.

10 Equivalents

- Those skilled in the art will know, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are
- 15 intended to be encompassed by the following claims.

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## CLAIMS

What is claimed is:

1. Method for treating or preventing a disease or medical disorder which can be ameliorated by delivery of NO or its biological equivalent to tissues affected by the disease or medical disorder, in an animal or human, comprising administering to the animal or human nitrosyl-heme-containing donors of NO.
2. The method of Claim 1 wherein the nitrosyl-heme-containing donor of NO is nitrosylhemoglobin.
3. Method for making stable nitrosyl-deoxyhemoglobin comprising adding NO to deoxyhemoglobin in an aqueous solution such that the ratio of added NO:heme is less than about 1:100 or greater than about 0.75.
4. Method for making SNO-oxyhemoglobin, comprising adding NO to an aqueous solution of oxyhemoglobin and buffer having a pK of at least about 9.4, at a concentration of approximately 10 mM to 200 mM, at pH 7.4.
5. Method for making nitrosyl-oxyhemoglobin comprising adding NO to oxyhemoglobin in an aqueous solution such that the ratio of NO:hemoglobin is less than about 1:30.
6. Hemoglobin conjugated to an NO-donor.
7. Hemoglobin of Claim 6, wherein the NO-donor is selected from the group consisting of:

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diazeniumdiolates, nitroprusside, nitroglycerin and nitrosothiol.

8. A composition comprising hemoglobin and one or more NO-donors.
- 5 9. A method for treating or preventing a disease or medical disorder which can be ameliorated by delivery of NO or its biological equivalent to tissues affected by the disease or medical disorder, in an animal or human, comprising administering a heme-based blood  
10 substitute and inhaled NO to the animal or human.
10. A method for delivering CO to the tissues in an animal or human, comprising administering CO-derivatized hemoglobin to the animal or human.
- 15 11. A method of treating or preventing a disease or medical disorder which can be ameliorated by delivery of NO or its biological equivalent to tissues affected by the disease or medical disorder in an animal or human, comprising administering CO-derivatized hemoglobin and a nitrosated hemoglobin to the animal  
20 or human.
12. Nitrosylhemoglobin conjugated to one or more electron acceptors.
13. Nitrosylhemoglobin of Claim 12, wherein the electron  
25 acceptor is selected from the group consisting of: superoxide dismutase, stable nitroxide radicals, and oxidized forms of nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, flavin

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adenine dinucleotide, flavin mononucleotide, ascorbate and dehydroascorbate.

14. A composition comprising nitrosylhemoglobin and one or more electron acceptors.
- 5 15. Hemoglobin conjugated to nitric oxide synthase.
16. Hemoglobin of Claim 15, wherein the nitric oxide synthase is nitric oxide synthase of neurons.
17. A composition comprising hemoglobin and nitric oxide synthase.
- 10 18. A method for making isolated erythrocytes comprising nitrosylhemoglobin, comprising incubating deoxygenated erythrocytes in a solution comprising NO.
19. Isolated erythrocytes comprising nitrosylhemoglobin.
- 15 20. A method for treating shock in an animal or human comprising administering hemoglobin  $\alpha$ -chains to the animal or human.
21. A method for treating or preventing a disease or medical disorder which can be ameliorated by delivery of NO or its biological equivalent to tissues affected by the disease or medical disorder, in an animal or human, comprising administering hemoglobin  $\beta$ -chains to the animal or human.
- 20 22. A method for measuring NO equivalents in S-nitrosohemoglobin and nitrosyl(FeII)-hemoglobin in

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blood comprising red blood cells, comprising the steps of:

- a) lysing the red blood cells of a blood sample;
- b) preparing a desalted protein fraction of the lysed red blood cells;
- c) subjecting a protein fraction to photolysis, thereby liberating nitric oxide from S-nitrosohemoglobin and nitrosyl (FeII)-hemoglobin; and
- d) quantitating the nitric oxide in the protein fraction by measuring a chemiluminescence signal generated by a chemical reaction between nitric oxide and ozone, thereby measuring NO equivalents in S-nitrosohemoglobin and nitrosyl(FeII)-hemoglobin in blood.

23. A method for assaying nitric oxide production in disease states, comprising the steps of:

- a) lysing the red blood cells of a blood sample;
- b) preparing a protein fraction of the lysed red blood cells;
- c) subjecting a protein fraction to photolysis, thereby liberating nitric oxide from S-nitrosohemoglobin and nitrosyl(FeII)-hemoglobin; and
- d) quantitating [the amount of] nitric oxide in the protein fraction by measuring a chemiluminescence signal generated by a chemical reaction between nitric oxide and ozone.

24. A method for assaying thiol-bound NO in S-nitrosohemoglobin in red blood cells, comprising:

- a) isolating washed red blood cells from blood;



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- b) lysing the red blood cells, thereby obtaining a lysate;
  - c) desalting the lysate;
  - d) dividing the lysate into an aliquot contacted  
5 with mercury ions in excess over protein concentration in the lysate, and leaving another aliquot untreated by mercury ions, thereby obtaining a mercury-treated aliquot and an untreated aliquot;
  - 10 e) exposing the mercury-treated aliquot and untreated aliquot to oxygen;
  - f) isolating a mercury-treated low molecular weight fraction and an untreated low molecular weight fraction from the respective aliquots of e);
  - 15 g) contacting the low molecular weight fractions of f) with excess low molecular weight thiol under acidic conditions, thereby producing S-nitrosothiol in each fraction;
  - 20 h) measuring NO liberated from S-nitrosothiol in the fractions of g) by photolysis-chemiluminescence; and
  - i) determining a quantity of thiol-bound NO in S-nitrosohemoglobin from a difference in measurements in h).
- 25 25. A method for assaying NO equivalents in S-nitrosohemoglobin and nitrosyl(FeII)-hemoglobin in purified hemoglobin, comprising measuring NO equivalents in the purified hemoglobin by photolysis-chemiluminescence, thereby measuring NO equivalents in  
30 S-nitrosohemoglobin and nitrosyl(FeII)-hemoglobin.

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26. A method for measuring NO equivalents in S-nitrosohemoglobin and nitrosyl(FeII)-hemoglobin in red blood cells, comprising:
- a) isolating washed red blood cells from blood;
  - 5 b) lysing the red blood cells, thereby obtaining a lysate;
  - c) desalting the lysate; and
  - d) measuring NO equivalents in the lysate of c) by photolysis-chemiluminescence, thereby measuring
  - 10 NO equivalents in S-nitrosohemoglobin and nitrosyl(FeII)-hemoglobin in red blood cells.
27. A method for measuring NO bound to nitrosyl(FeII)-hemoglobin in red blood cells, comprising:
- a) making a protein fraction from the red blood
  - 15 cells;
  - b) treating the protein fraction with excess  $\text{HgCl}_2$ , followed by exposure to air; and
  - c) subjecting the protein fraction of b) to photolysis of the NO ligand of nitrosyl(FeII)-
  - 20 hemoglobin followed by detection of NO by chemiluminescence, thereby measuring NO bound to nitrosyl(FeII)-hemoglobin.
28. A method for assaying S-nitrosohemoglobin comprising:
- a) isolating washed red blood cells from blood;
  - 25 b) lysing the red blood cells, thereby obtaining a lysate;
  - c) desalting the lysate;
  - d) contacting an aliquot of the lysate of c) with mercury ions in excess over protein
  - 30 concentration, thereby obtaining a mercury-treated aliquot and an untreated aliquot;

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- e) exposing the mercury-treated aliquot and the untreated aliquot to oxygen;
  - f) measuring NO equivalents in the mercury-treated aliquot of e) and NO equivalents in the untreated aliquot of e) by photolysis-chemiluminescence; and
  - g) determining a quantity of S-nitrosohemoglobin from the NO equivalents measured in f).
29. A method for measuring S-nitrosohemoglobin and nitrosyl(FeII)-hemoglobin in red blood cells, comprising:
- a) isolating washed red blood cells from blood;
  - b) lysing the red blood cells, thereby obtaining a lysate;
  - c) desalting the lysate; and
  - d) measuring NO equivalents from the lysate of c) by photolysis-chemiluminescence.
30. A method for assaying nitrosyl(Fe)-hemoglobin comprising:
- a) isolating washed red blood cells from blood;
  - b) lysing the red blood cells, thereby obtaining a lysate;
  - c) desalting the lysate;
  - d) contacting an aliquot of the lysate with mercury ions in excess over protein concentration, thereby obtaining a mercury-treated aliquot and an untreated aliquot;
  - e) exposing the mercury-treated aliquot and untreated aliquot to oxygen;
  - f) measuring NO equivalents in the aliquots of e) by photolysis-chemiluminescence, thereby obtaining

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- 1) a quantity for S-nitrosohemoglobin and 2) a quantity for nitrosyl(FeII)-hemoglobin and S-nitrosohemoglobin combined, respectively; and
- g) determining a quantity for nitrosyl(FeII)-hemoglobin by subtracting 1) from 2).
- 5
31. A method for assessing oxygen delivery to a site in the body, comprising determining S-nitrosohemoglobin and nitrosylhemoglobin values in a blood sample from the site, and using said values to assess oxygen
- 10 delivery.
32. The method of Claim 31, further comprising determining an oxygen value in the blood sample and using said value to assess oxygen delivery.

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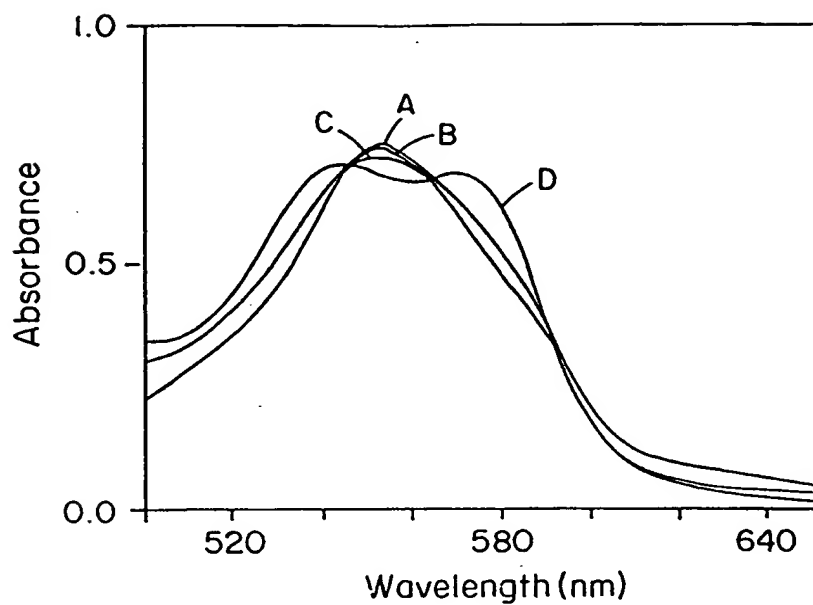


FIG. 1A

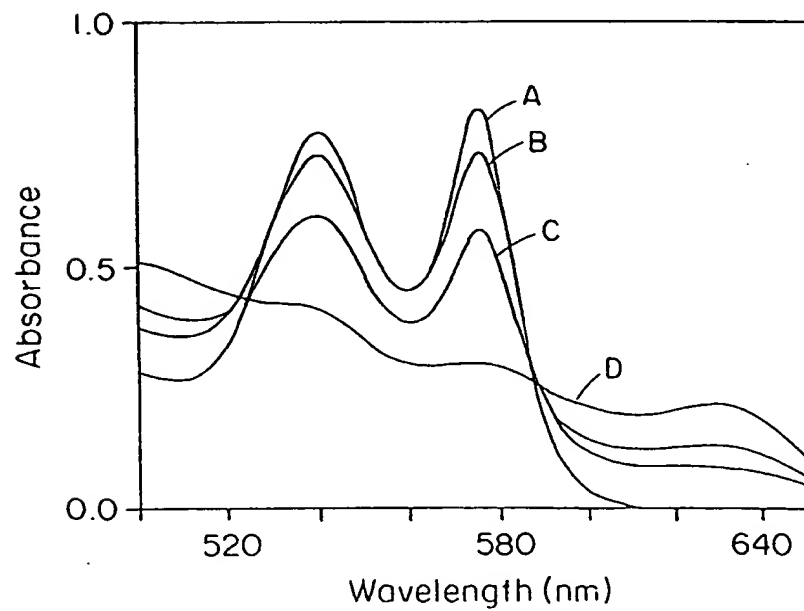


FIG. 1B

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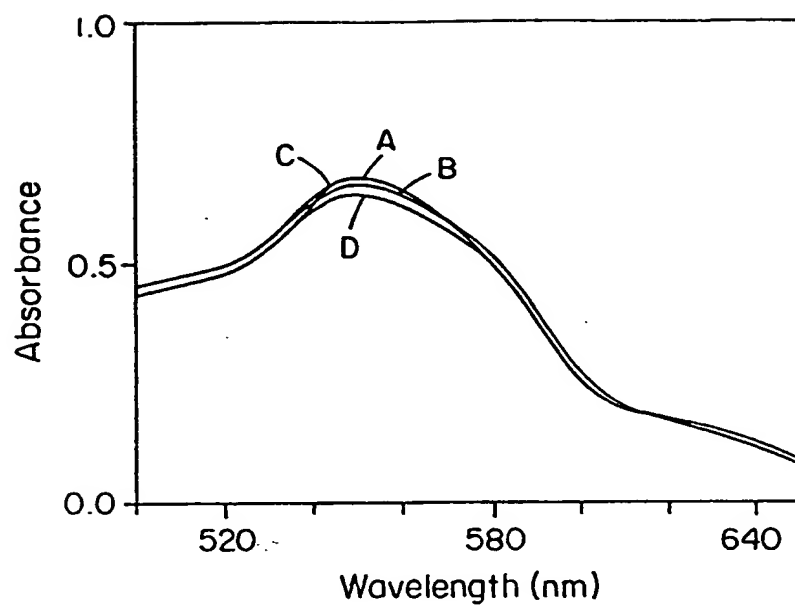


FIG. IC

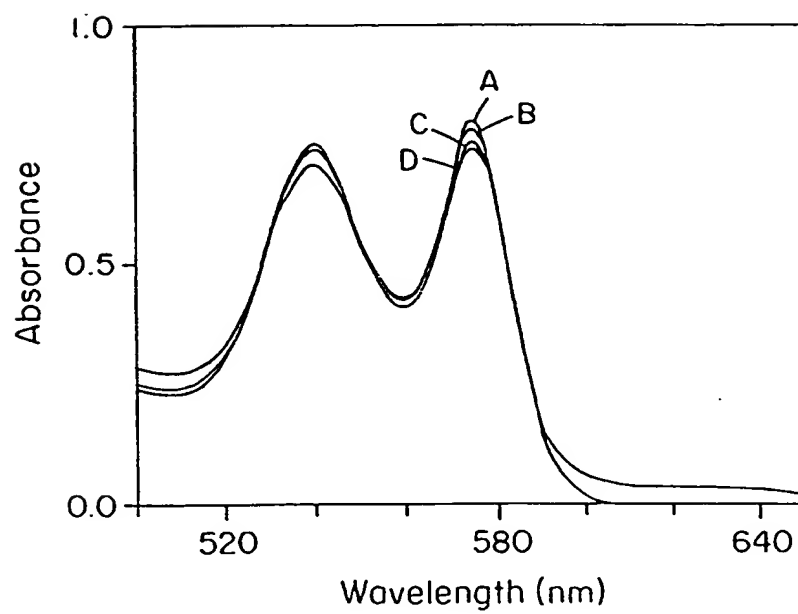


FIG. ID

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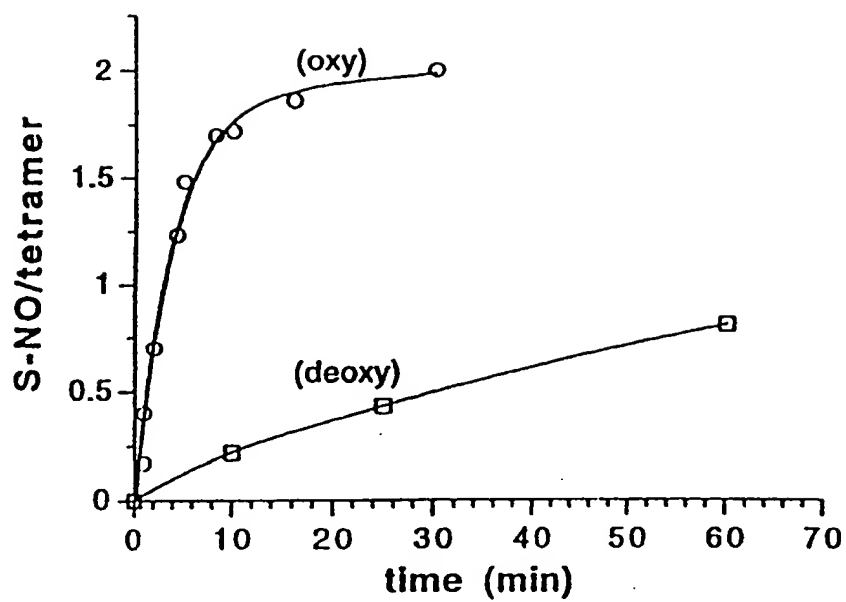


FIG. 2A

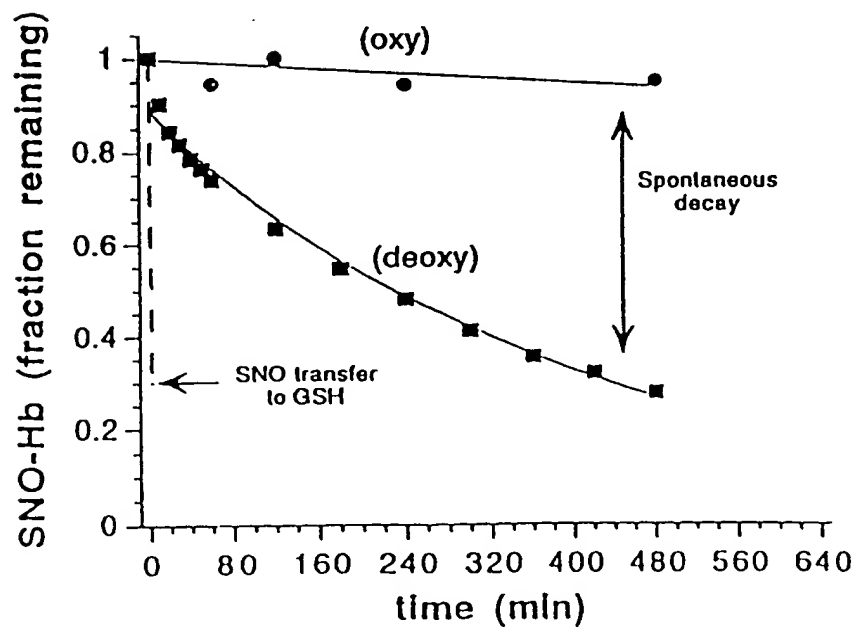


FIG. 2B

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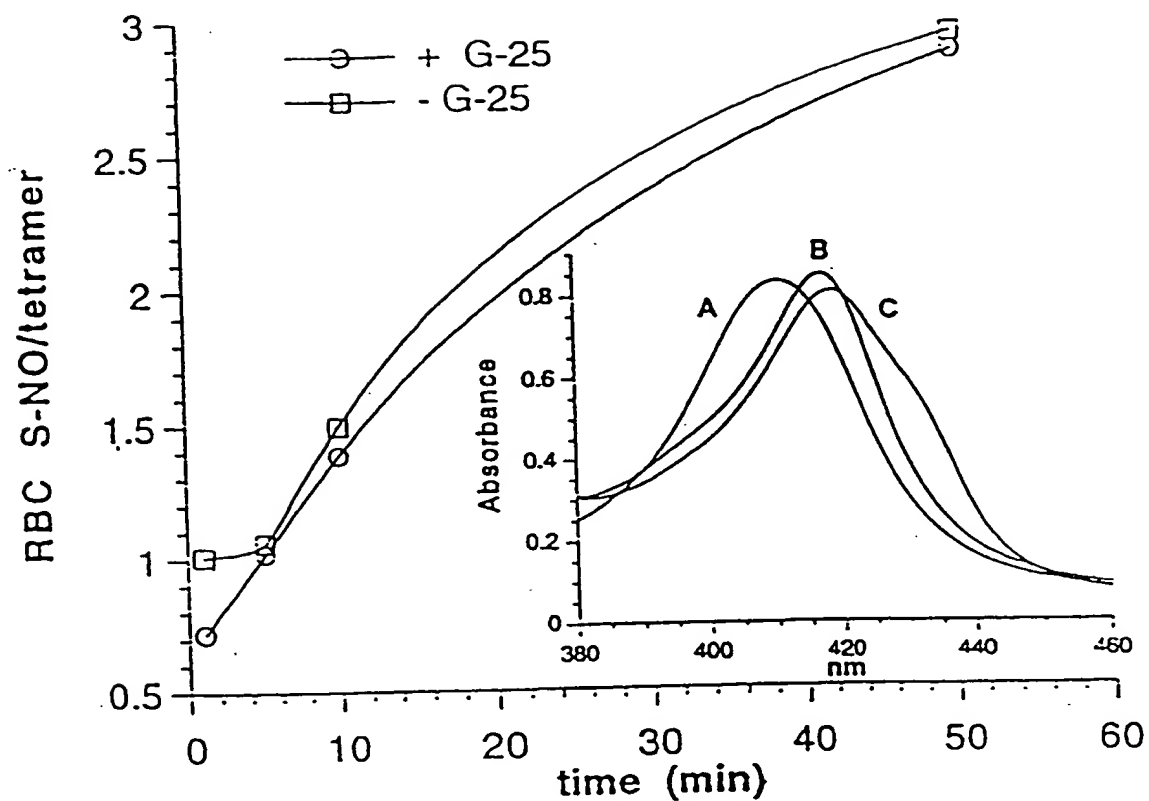


FIG. 3A

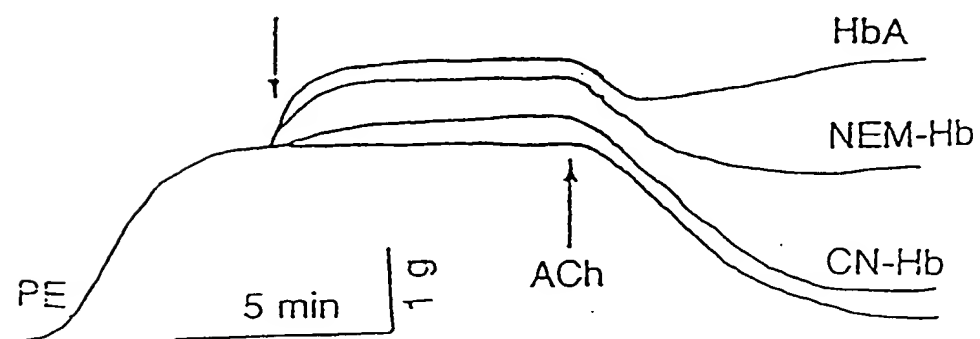


FIG. 3B



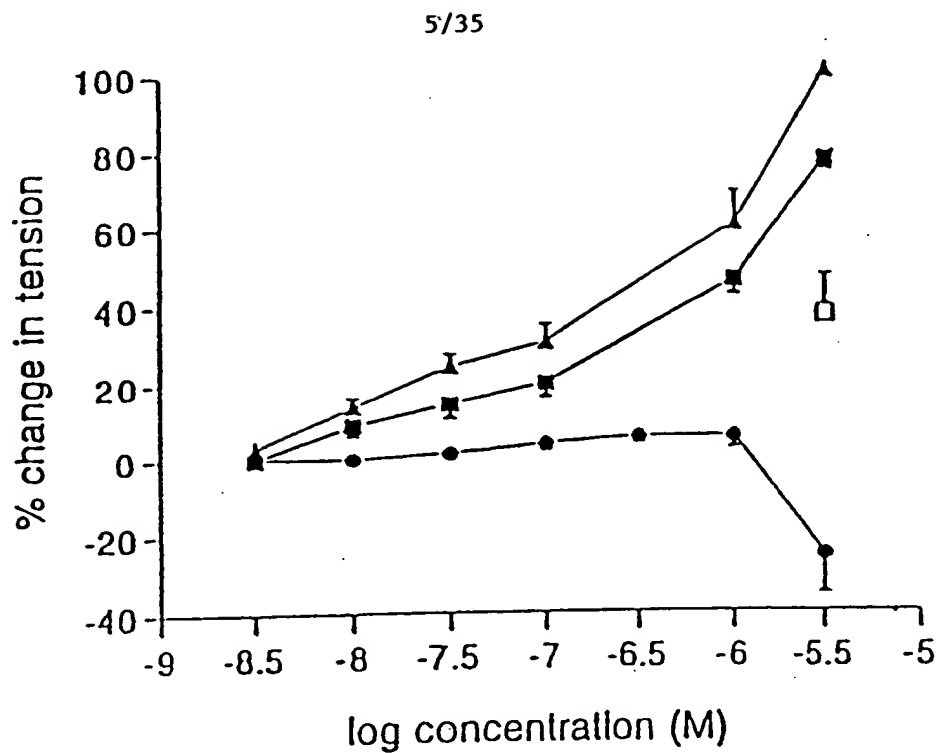


FIG. 4A

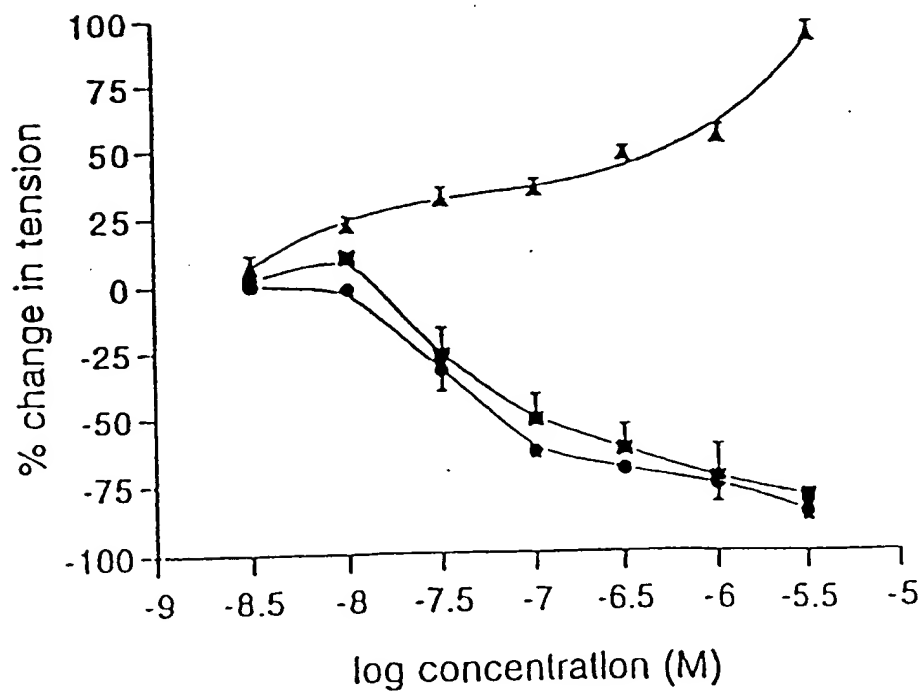


FIG. 4B

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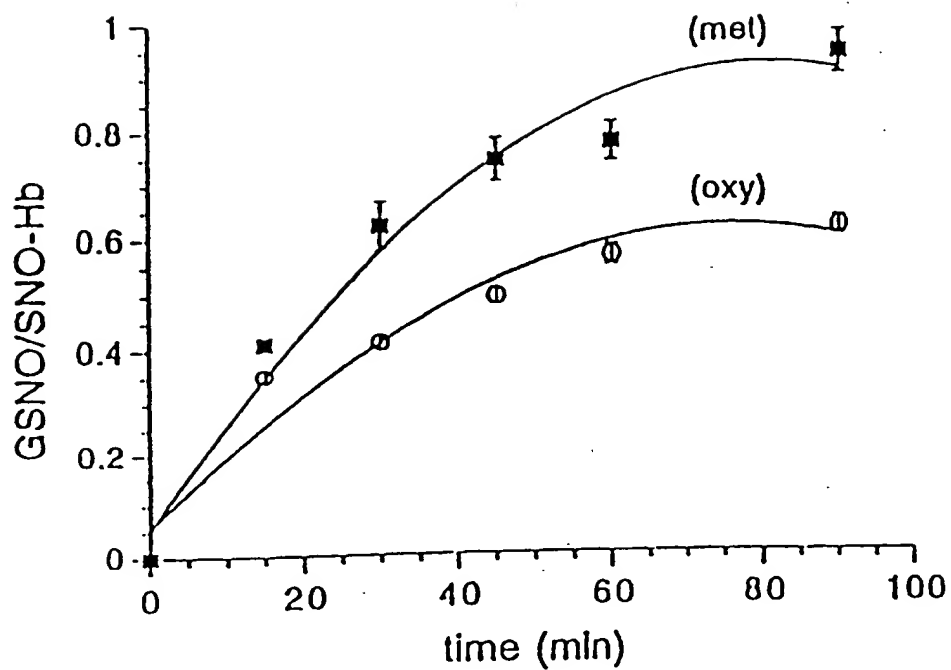


FIG. 4C

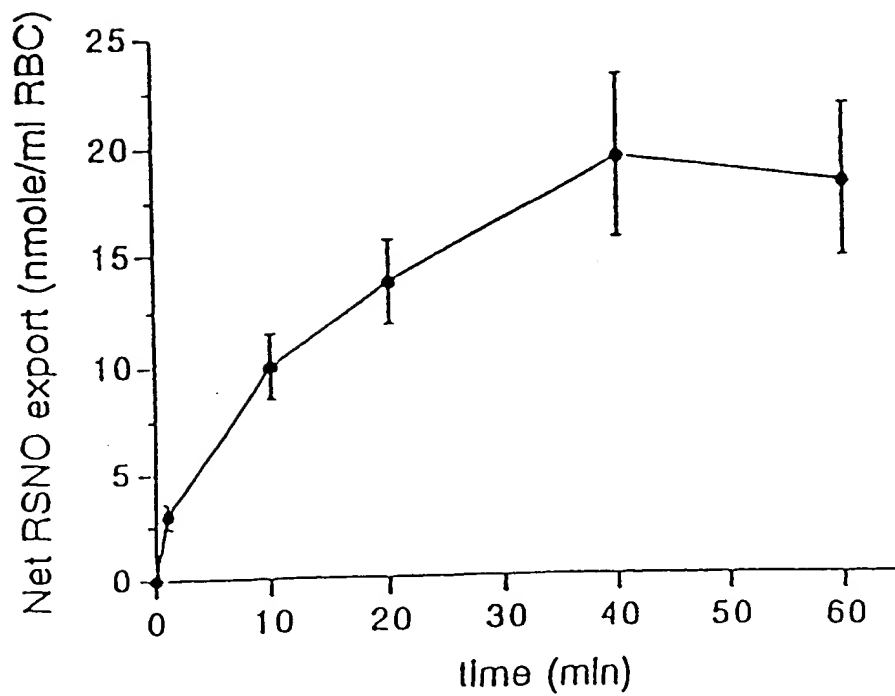


FIG. 4D

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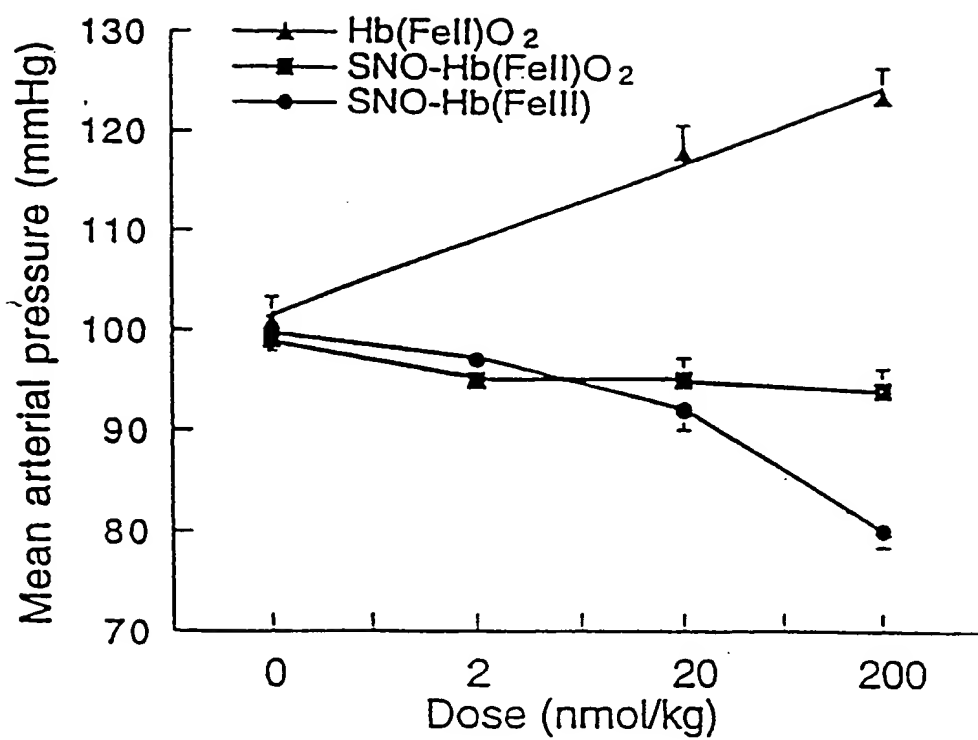
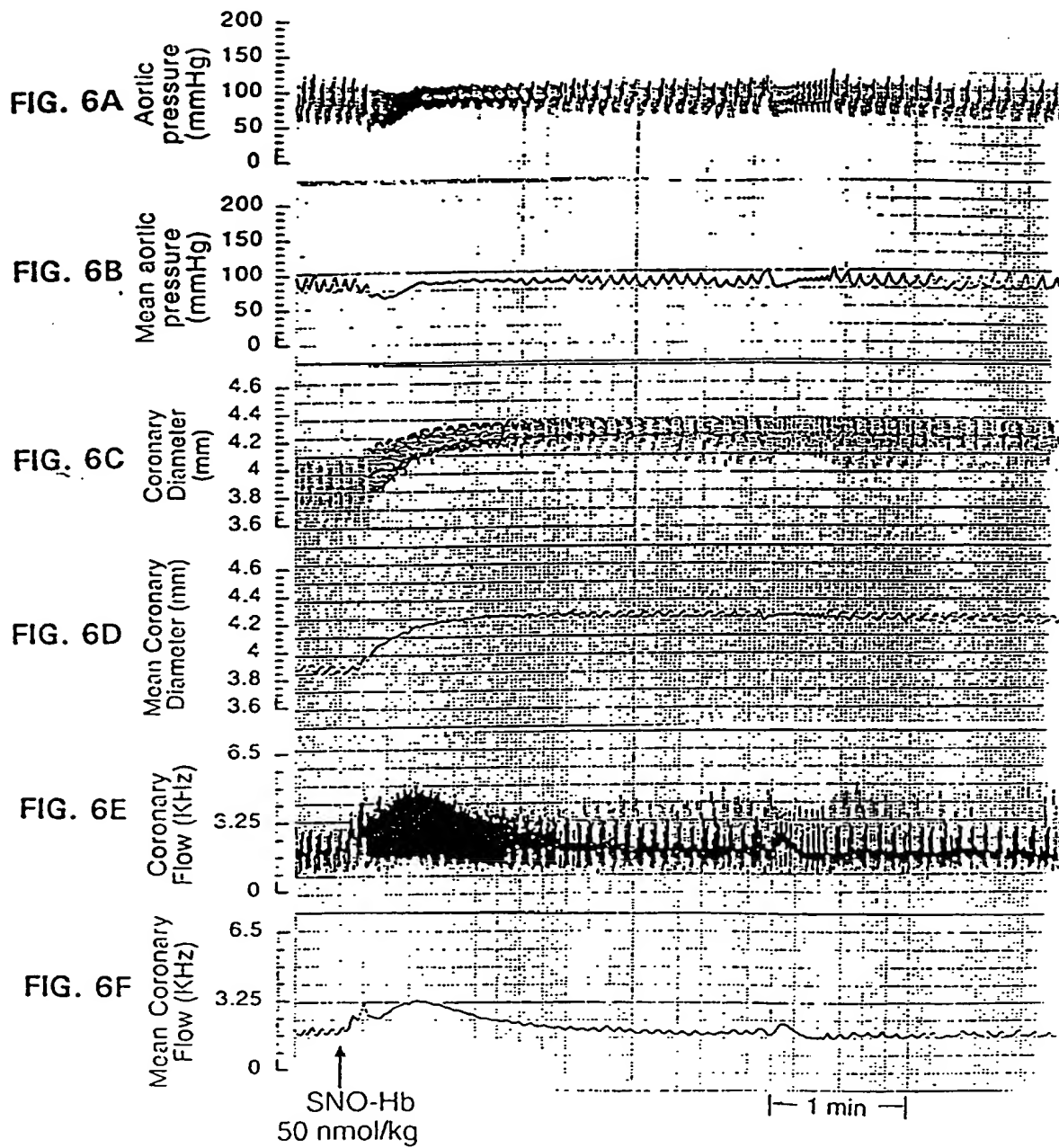
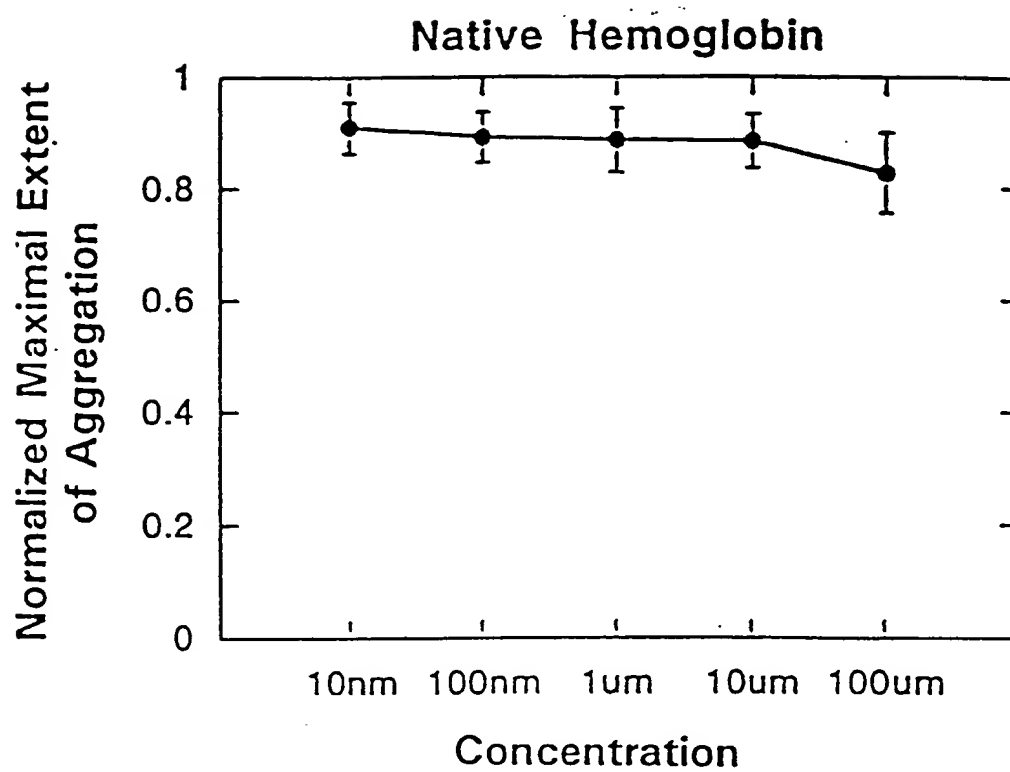


FIG. 5

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**FIG. 7A**

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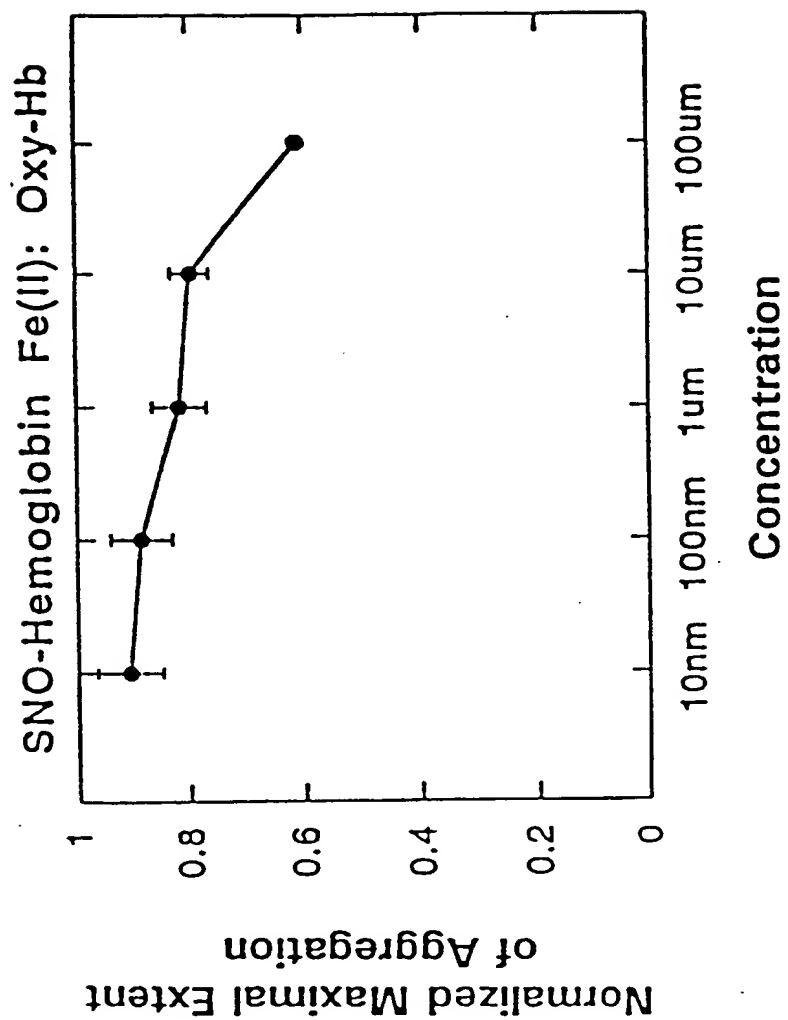


FIG. 7B

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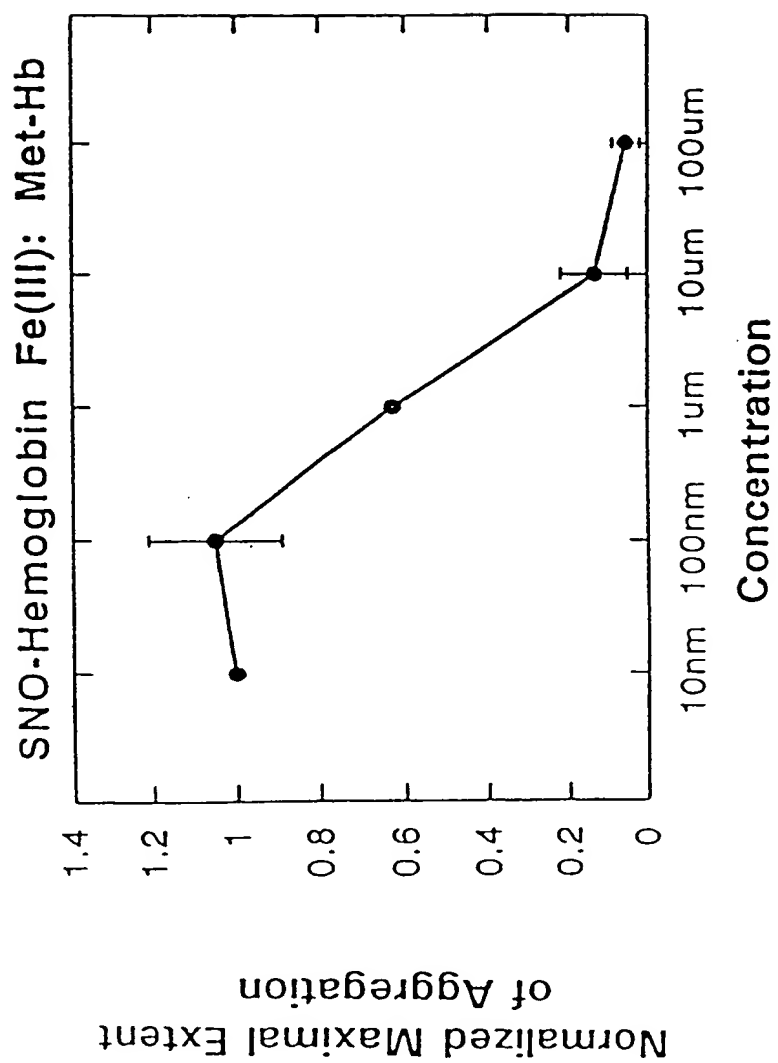


FIG. 7C

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(cGMP) Under the Effect of Various Types of Hemoglobin

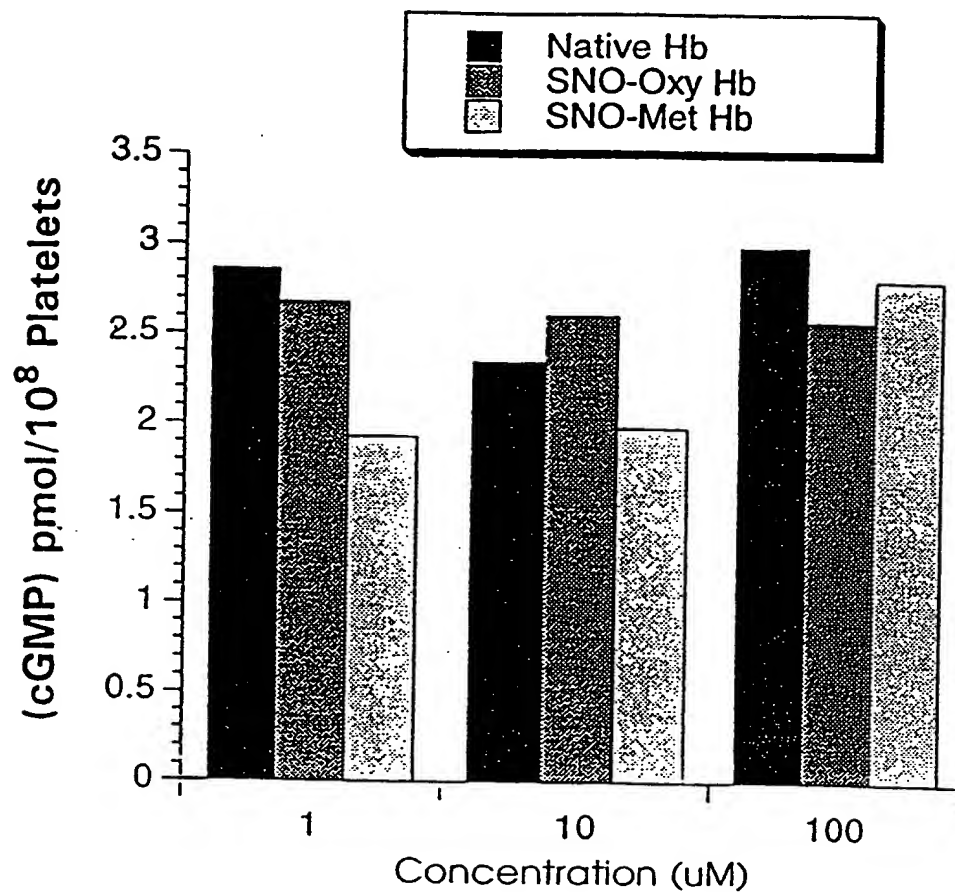


FIG. 8



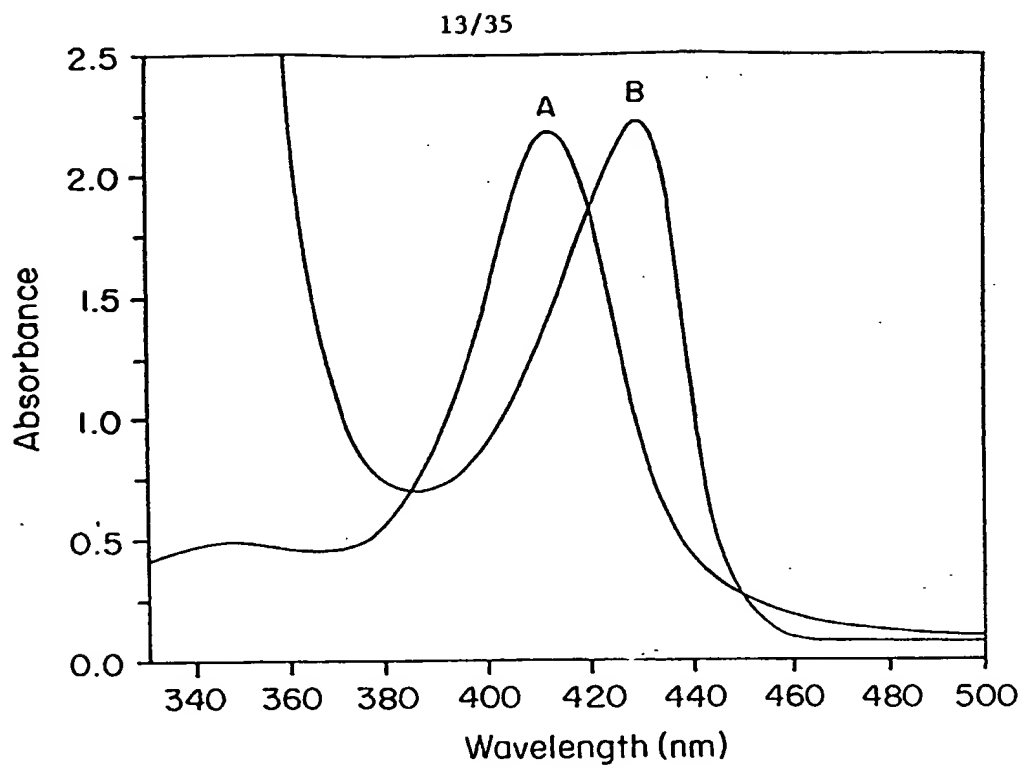


FIG. 9A

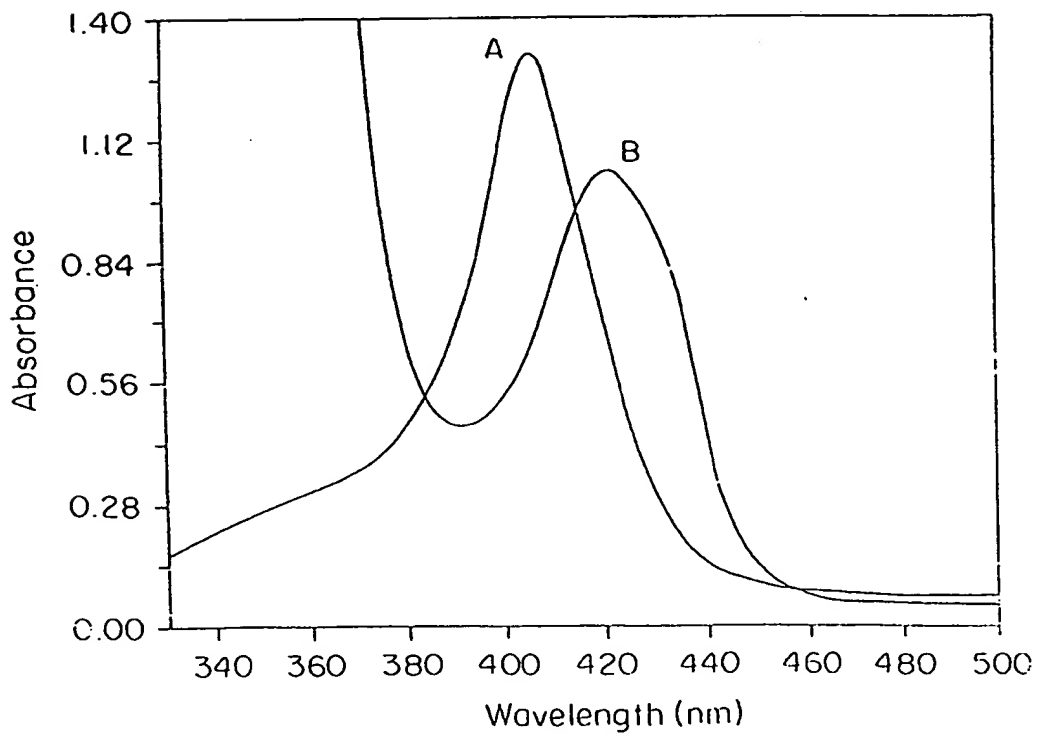


FIG. 9B

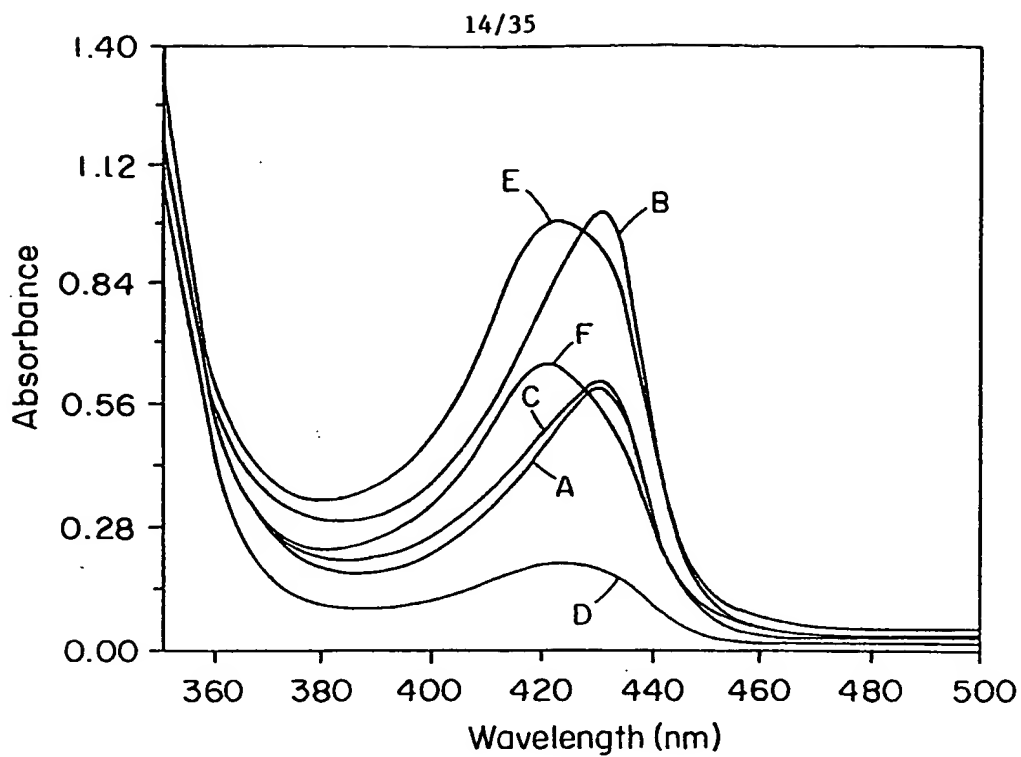


FIG. 9C

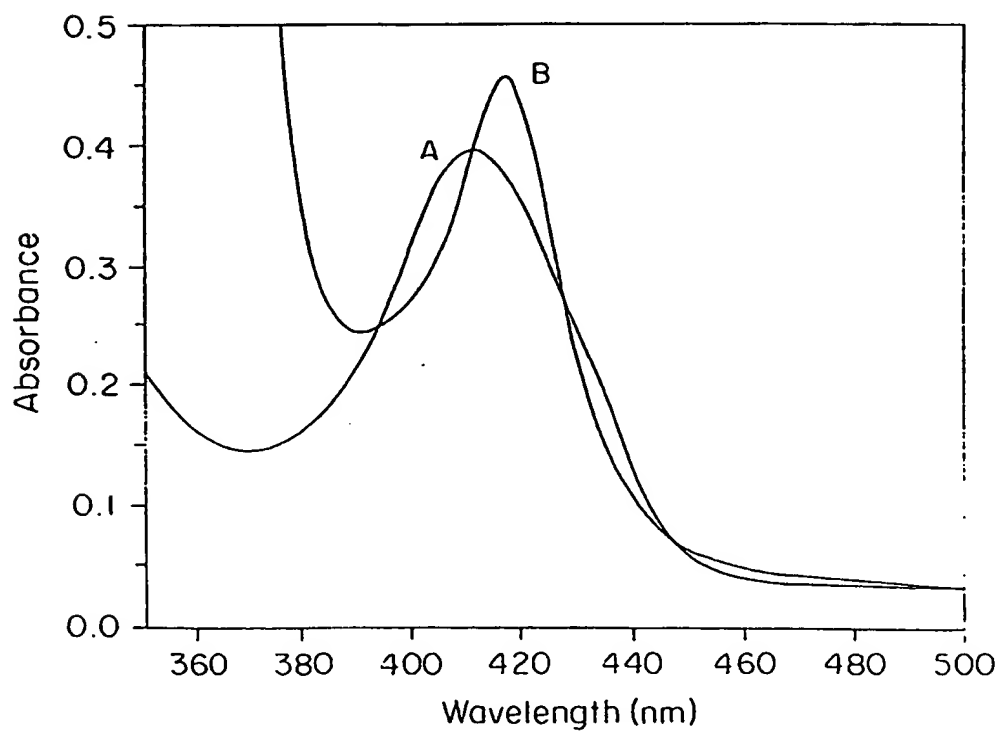


FIG. 9D

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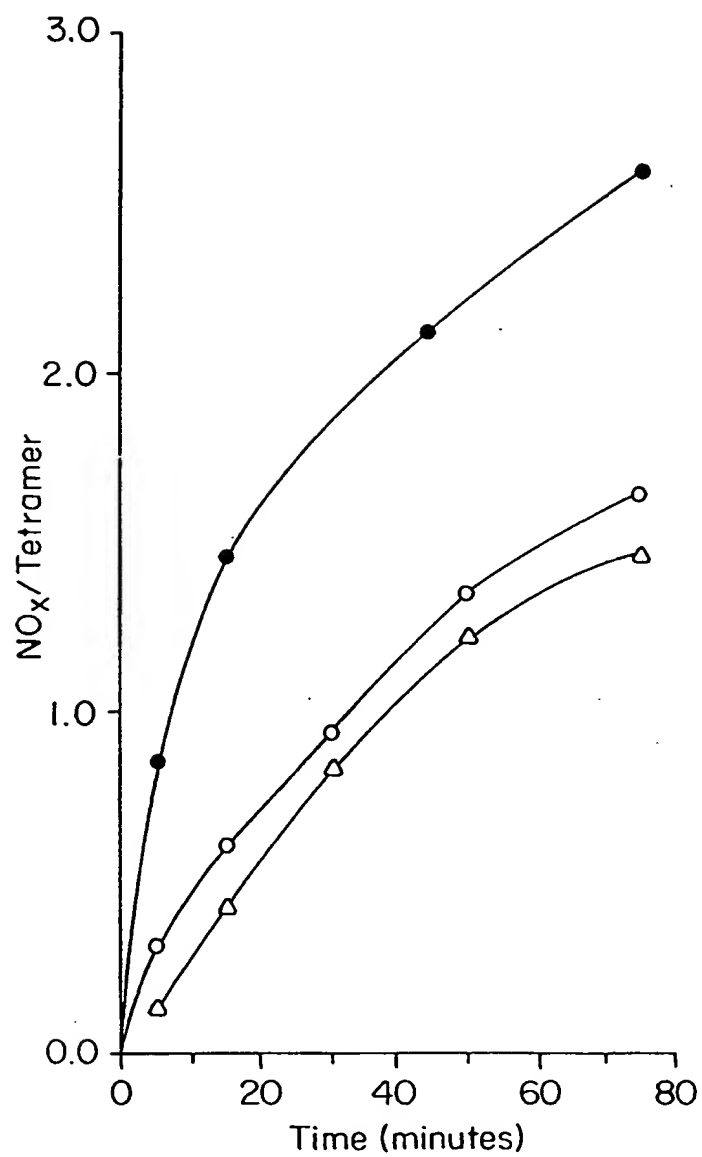


FIG. 9E

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Change in Blood Flow in Rat Caudatoputamen Nucleus  
after Injecting SNO-Hb to Rats Breathing in 21% O<sub>2</sub>

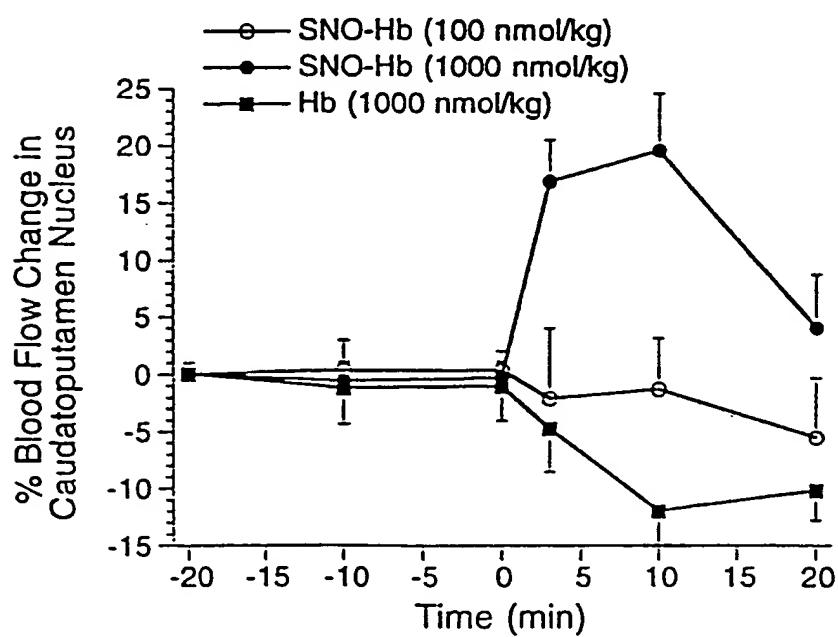


FIG. 10

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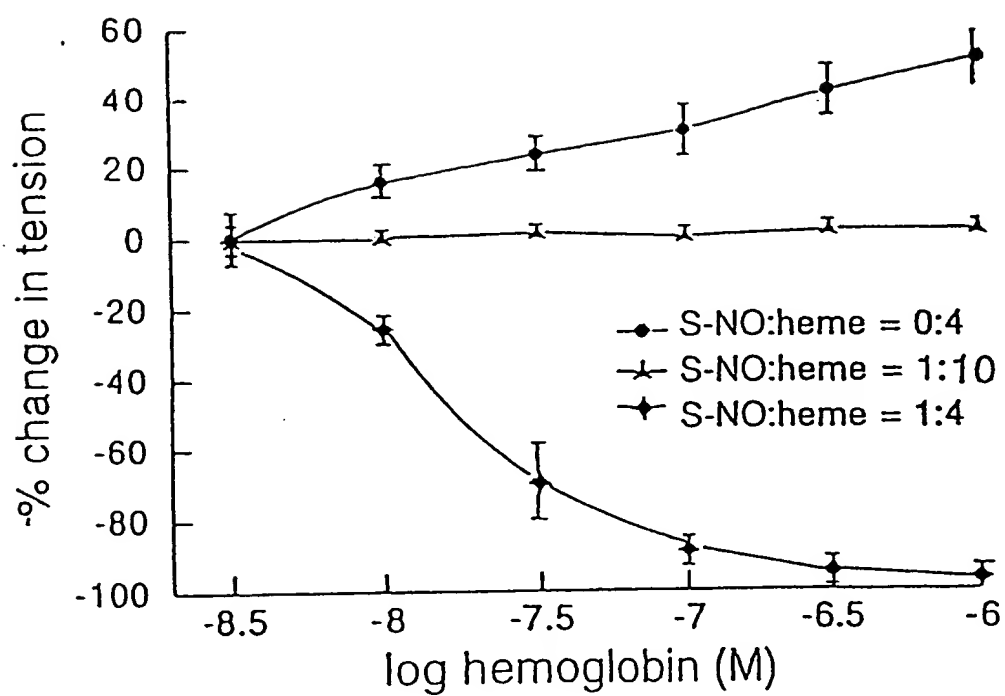
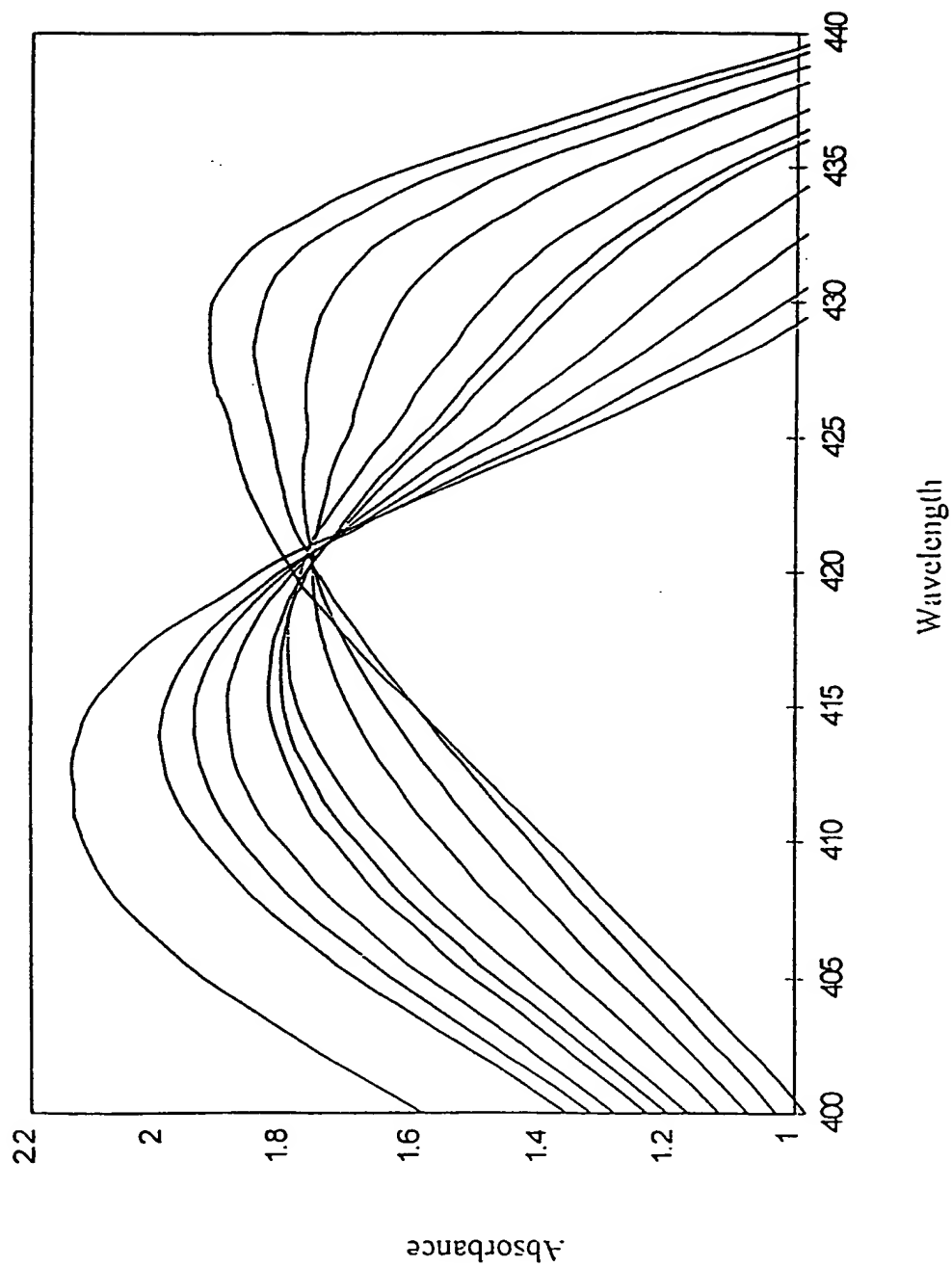


FIG. 11

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FIG. 12



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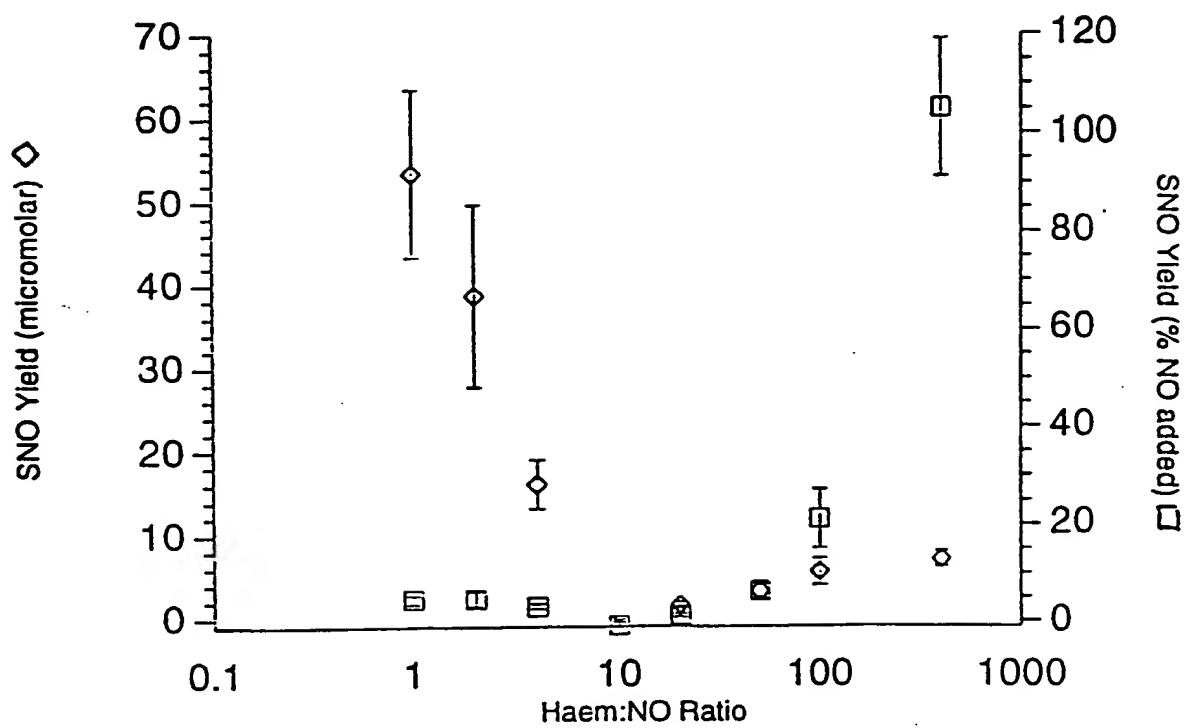


FIG. 13





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FIG. 14B

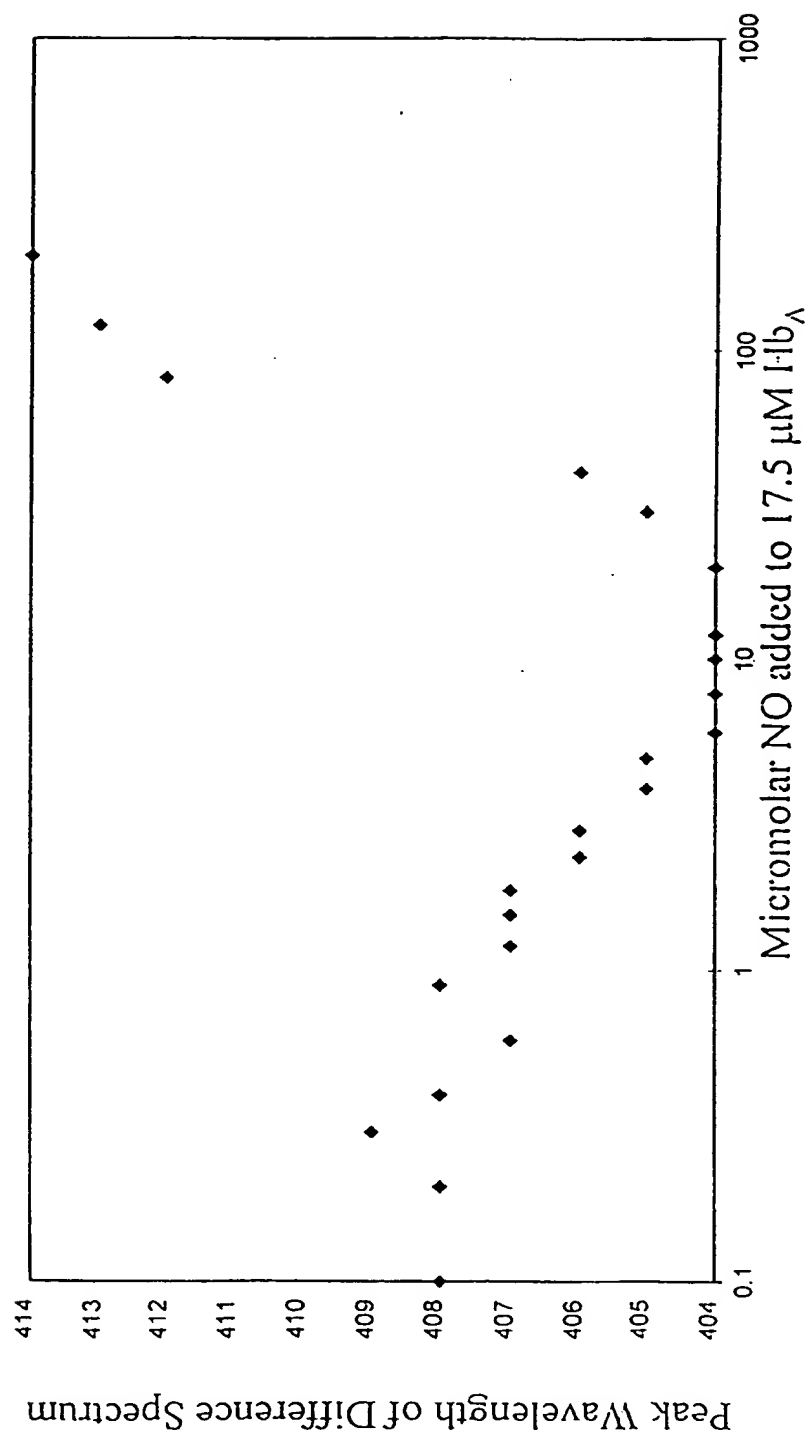
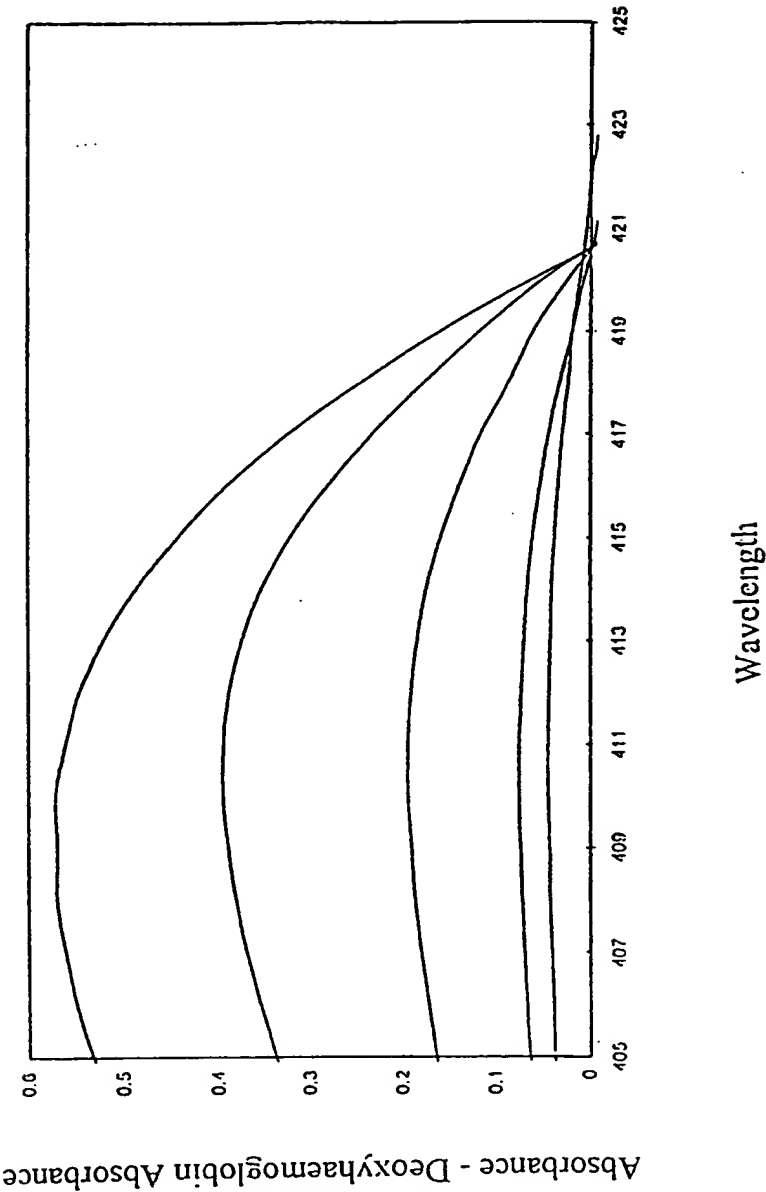
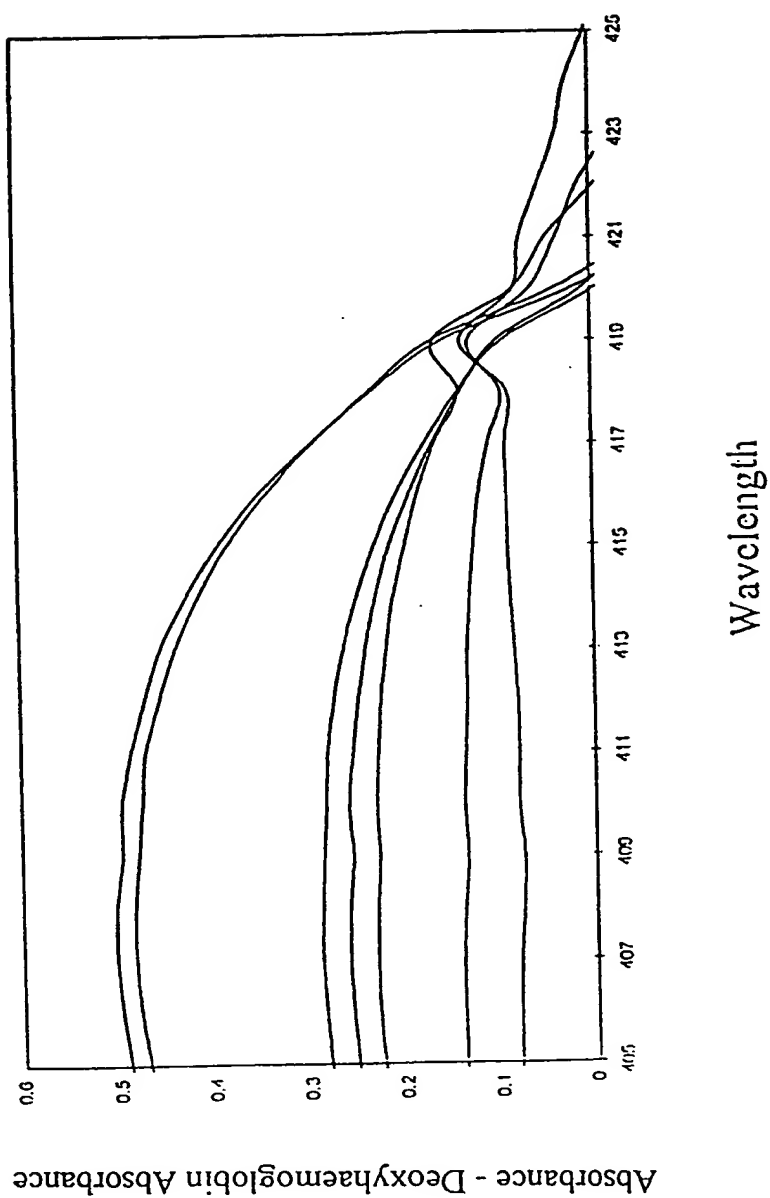


FIG. 15A



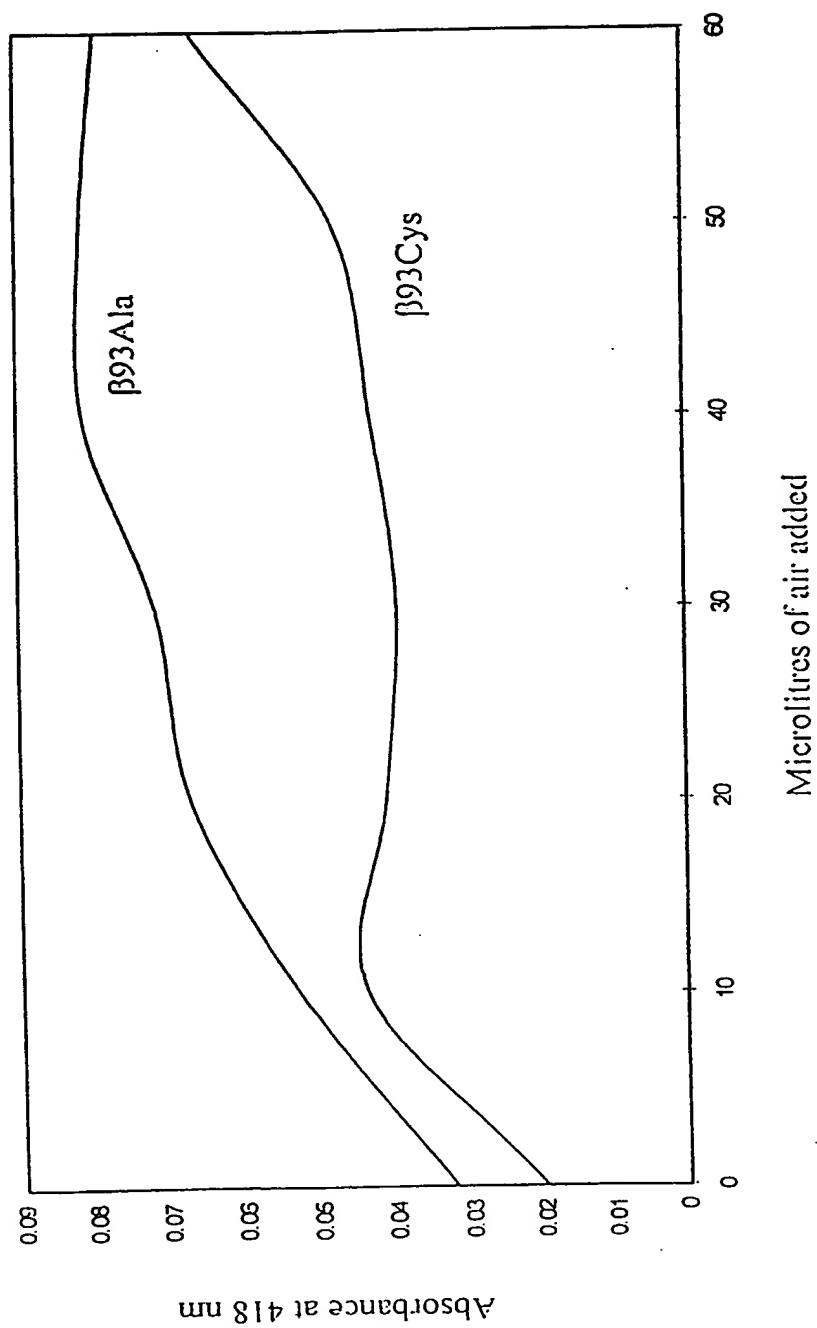
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FIG. 15B



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FIG. 16



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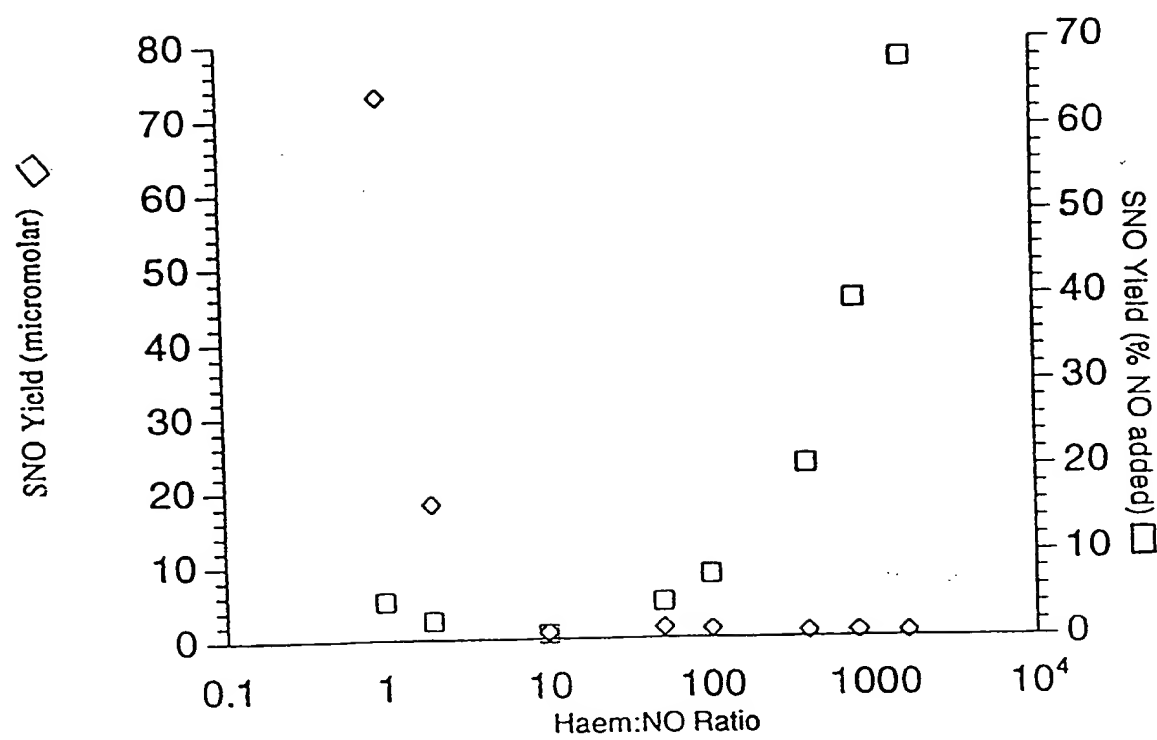
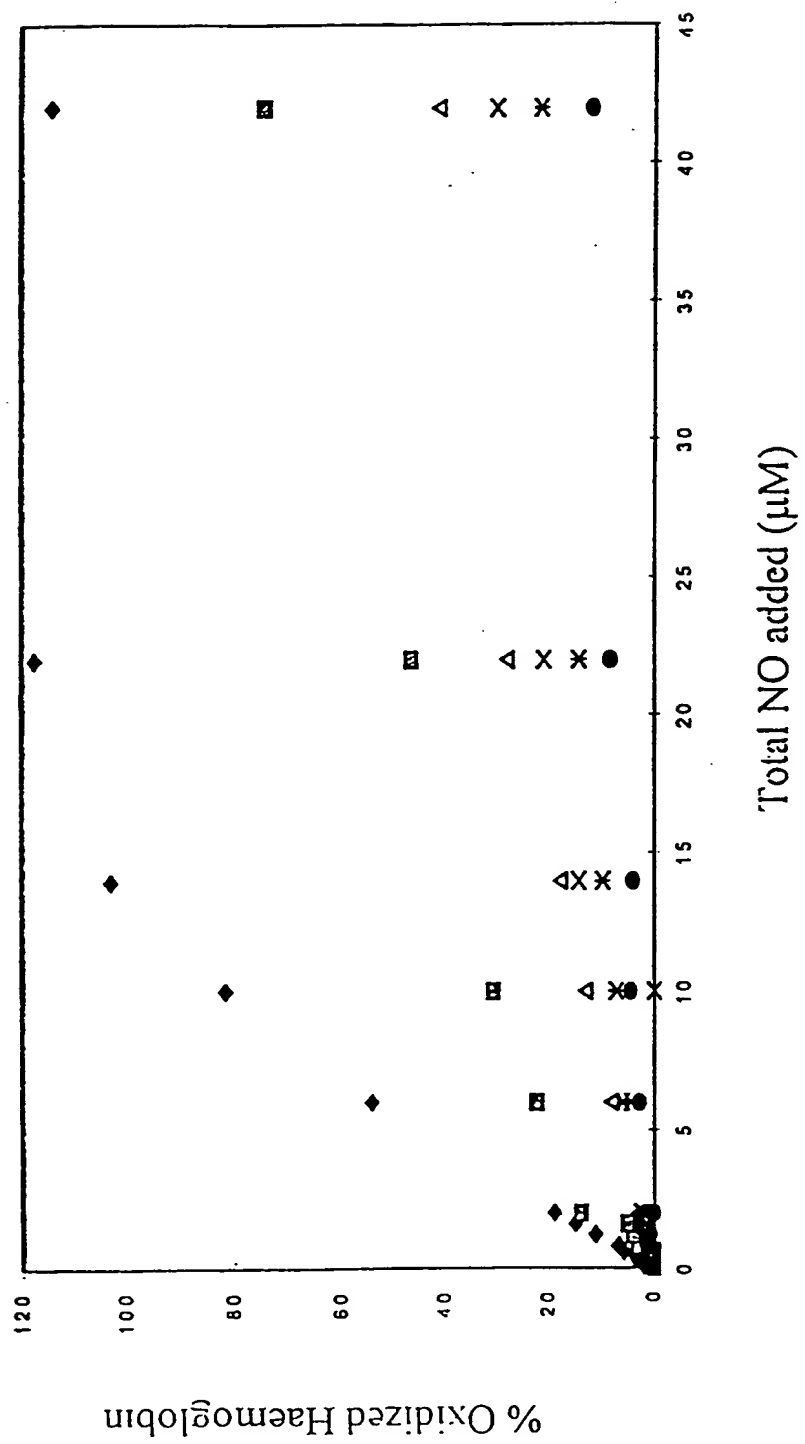


FIG. 17

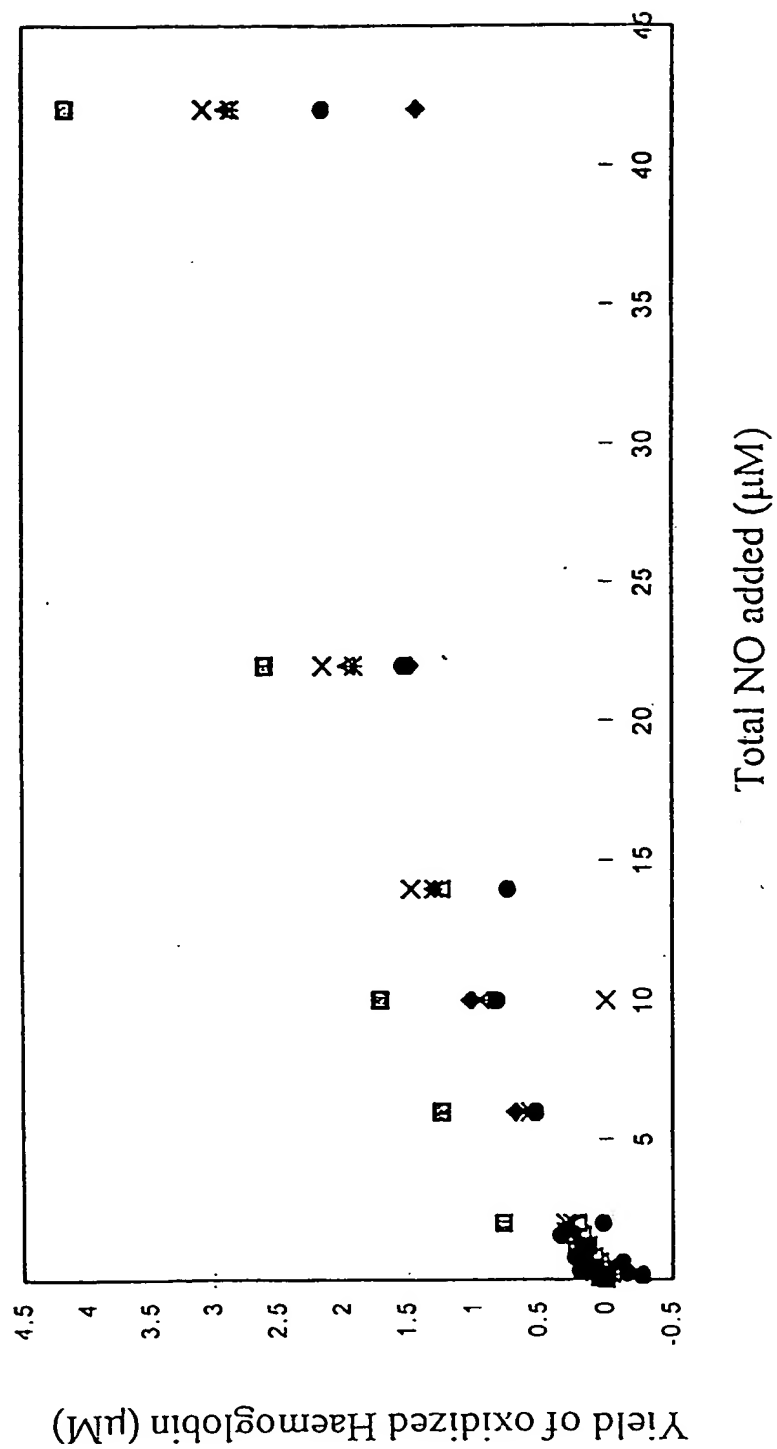
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FIG. 18A



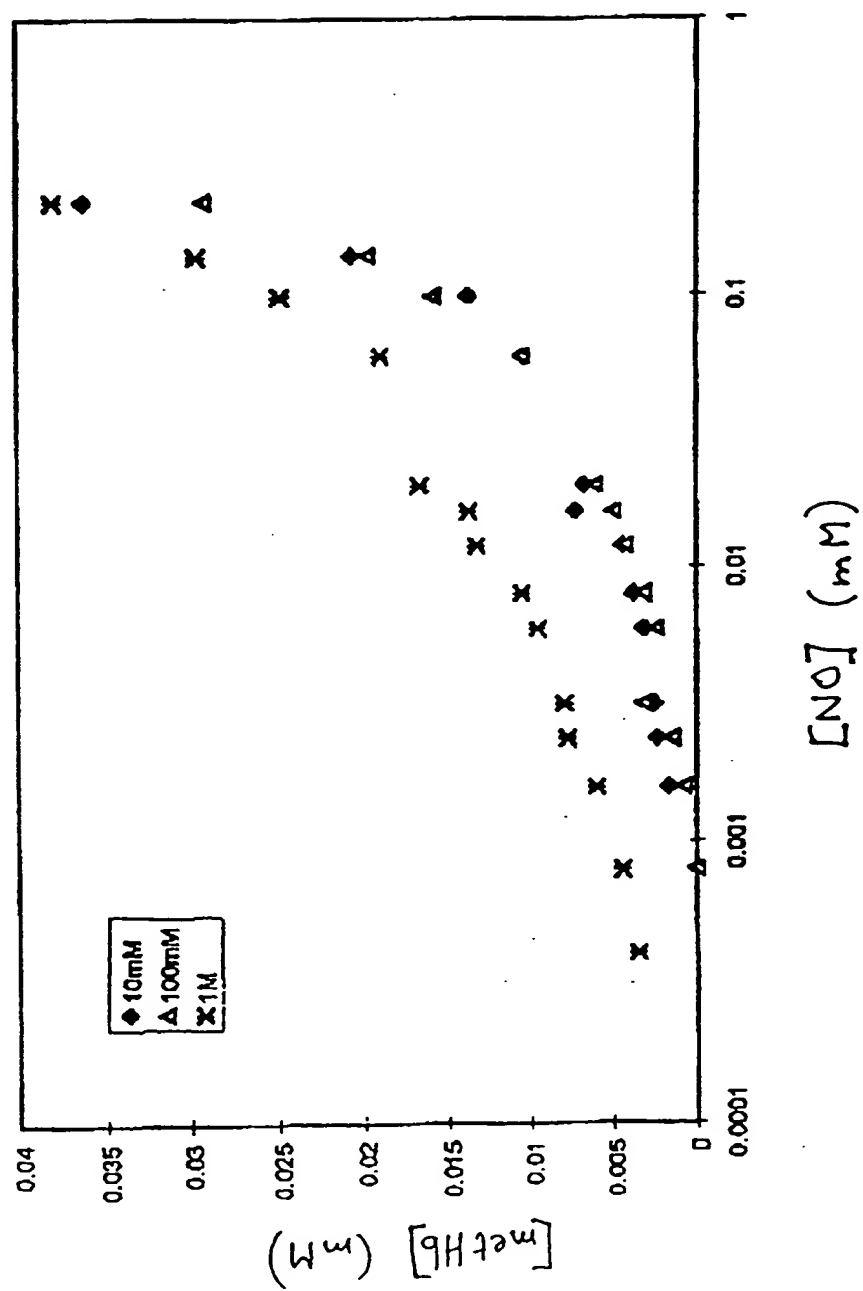
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FIG. 18B



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FIG. 19





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FIG. 20B

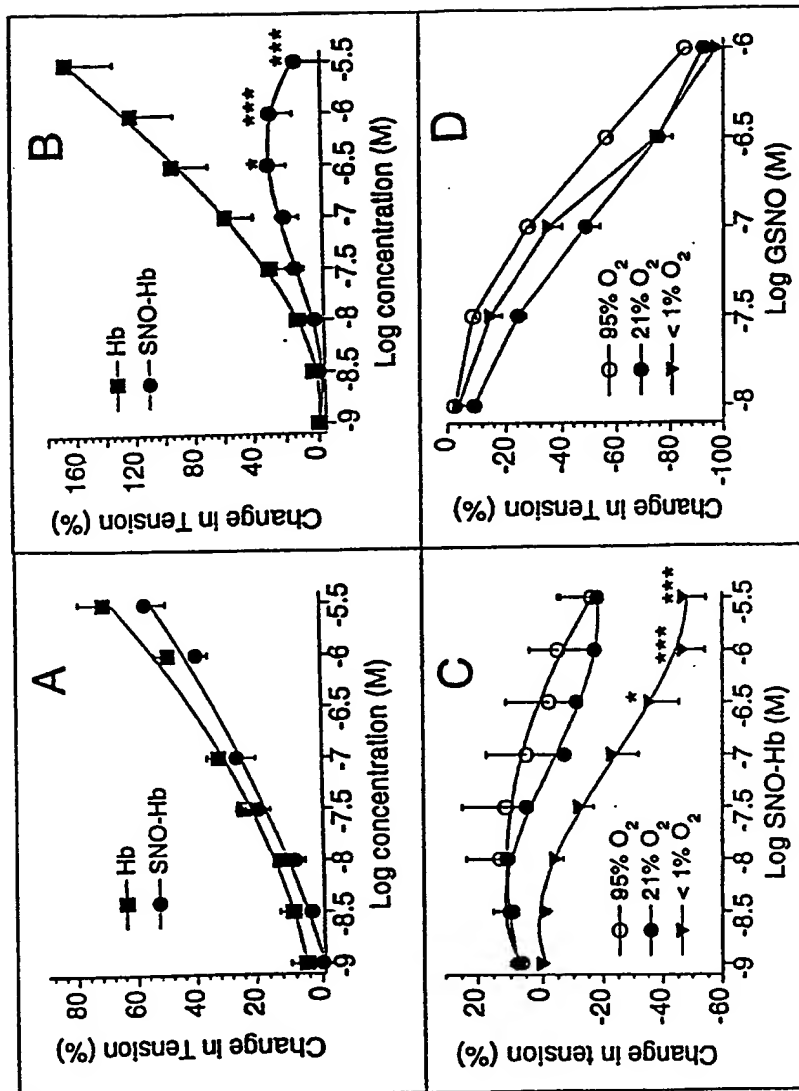


FIG. 20A

FIG. 20D

FIG. 20C

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FIG. 21A

FIG. 21B

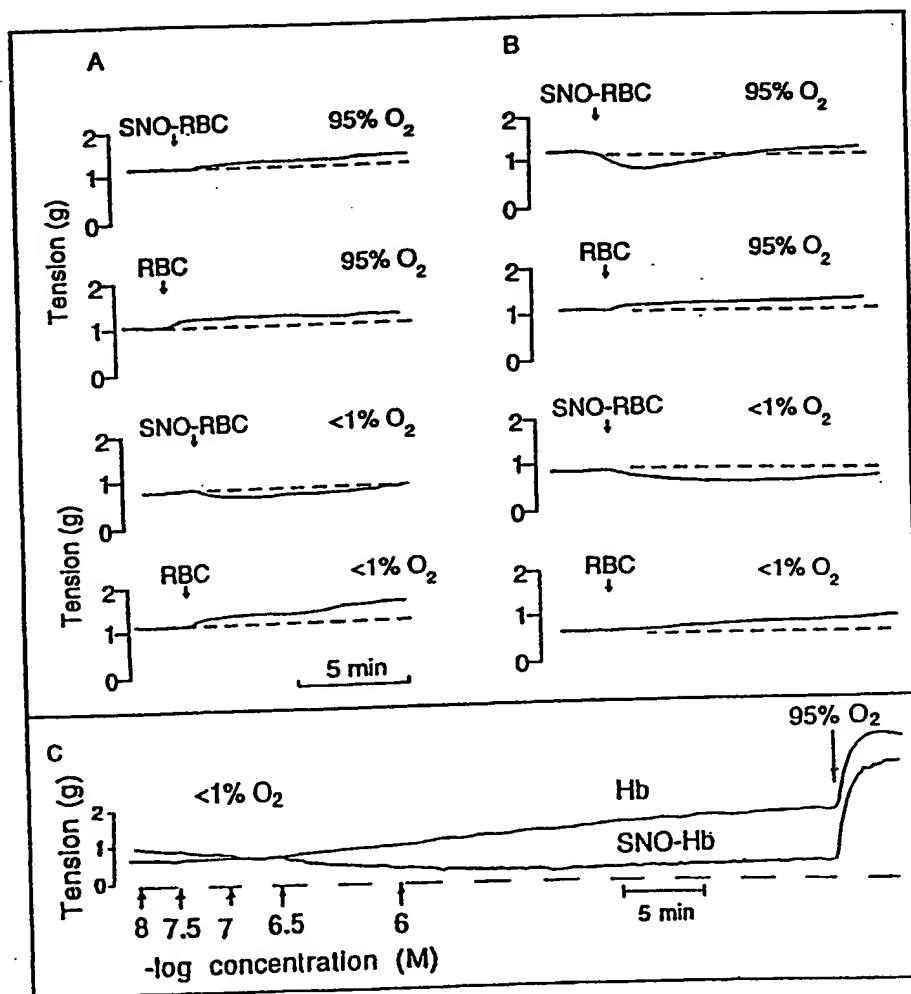
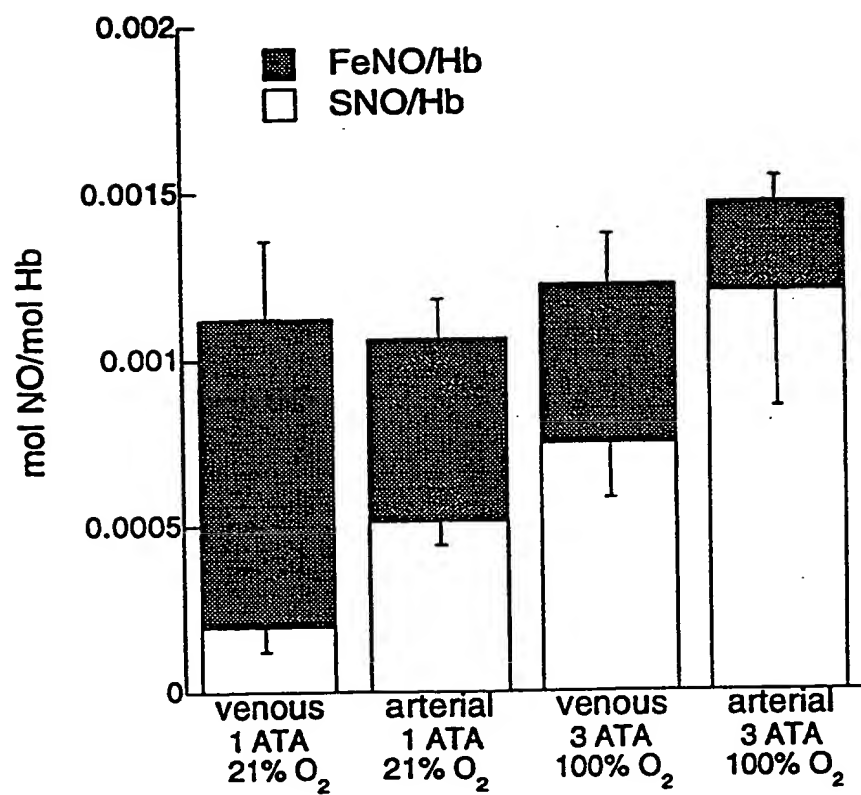


FIG. 21C

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FIG. 22

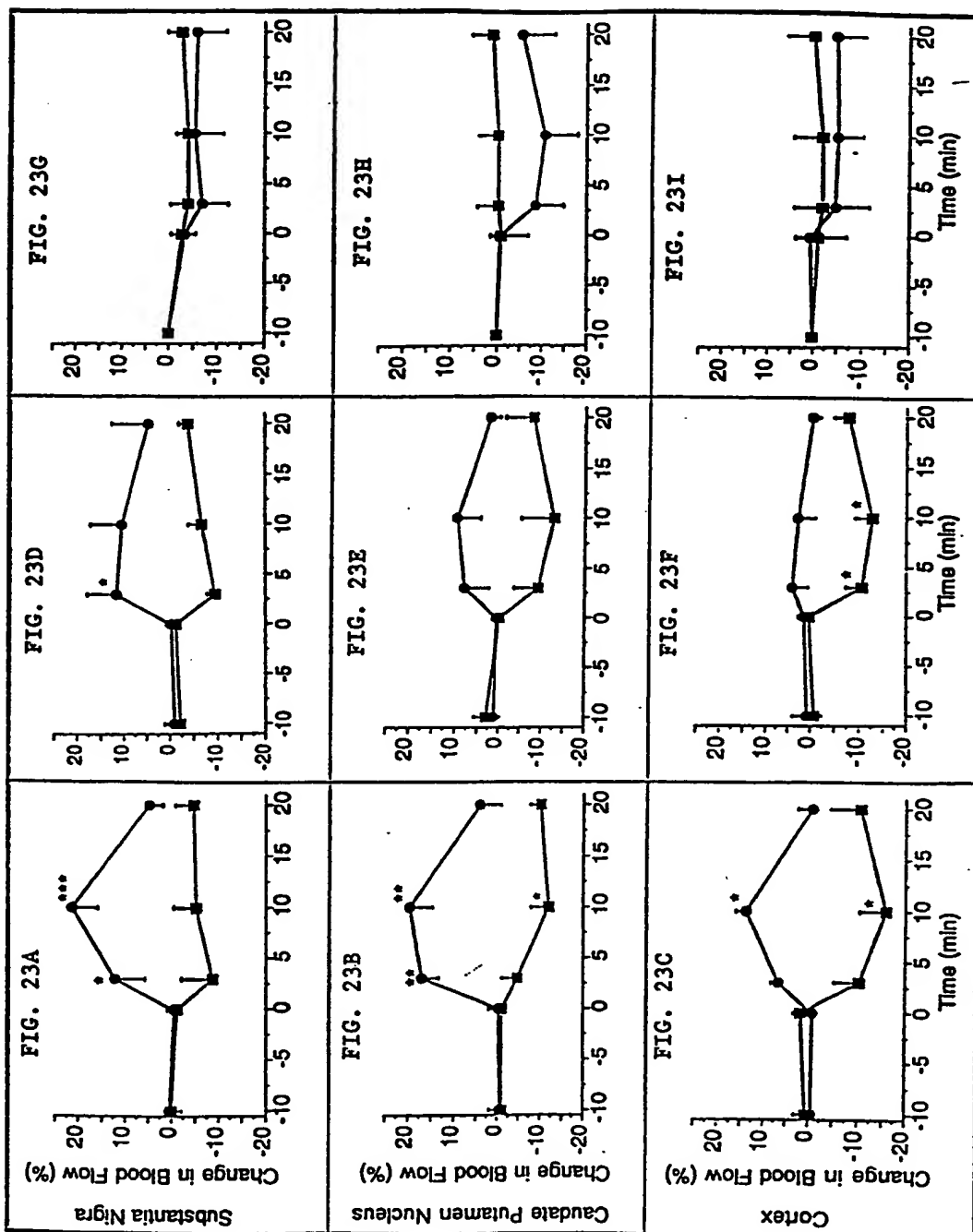


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100% O<sub>2</sub>  
3 ATA

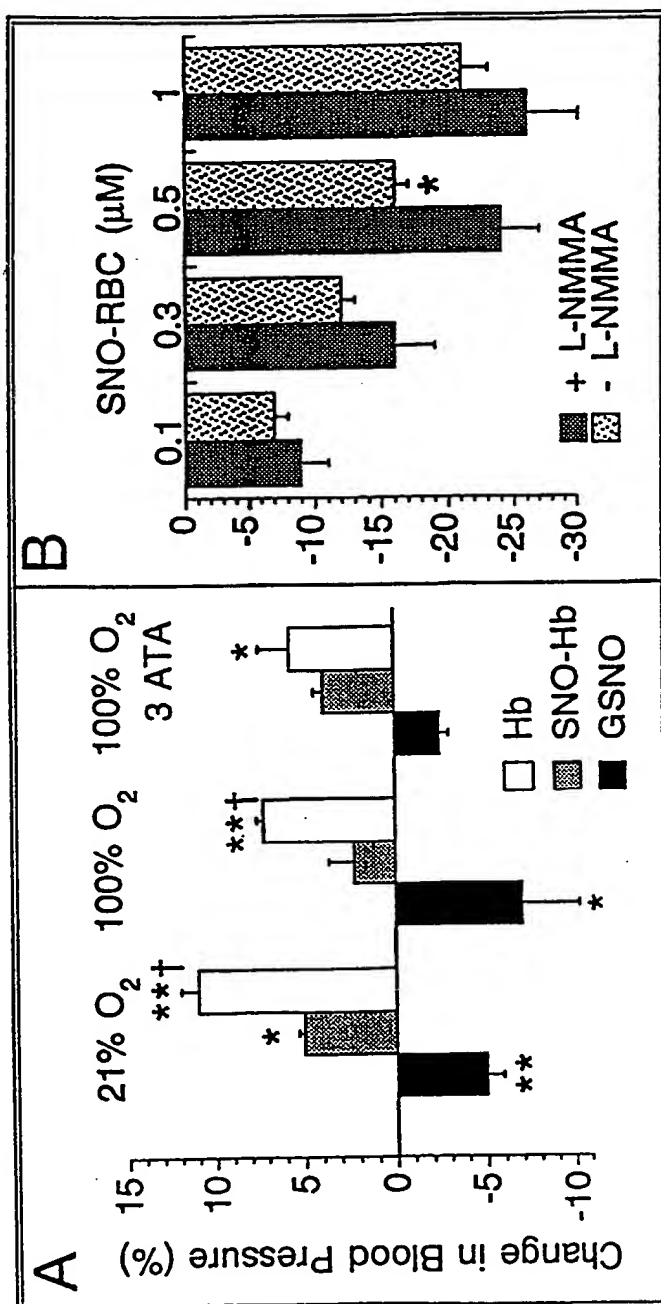
100% O<sub>2</sub>

21% O<sub>2</sub>



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FIG. 24B



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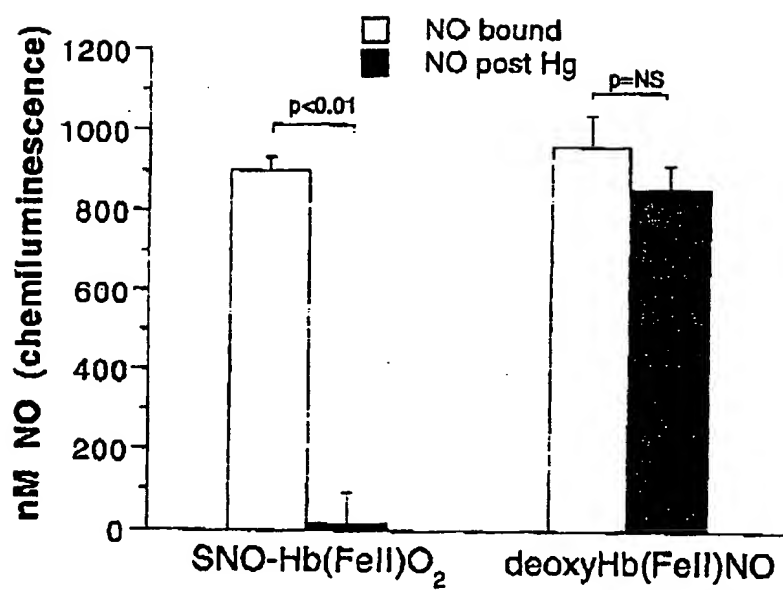


Fig. 25

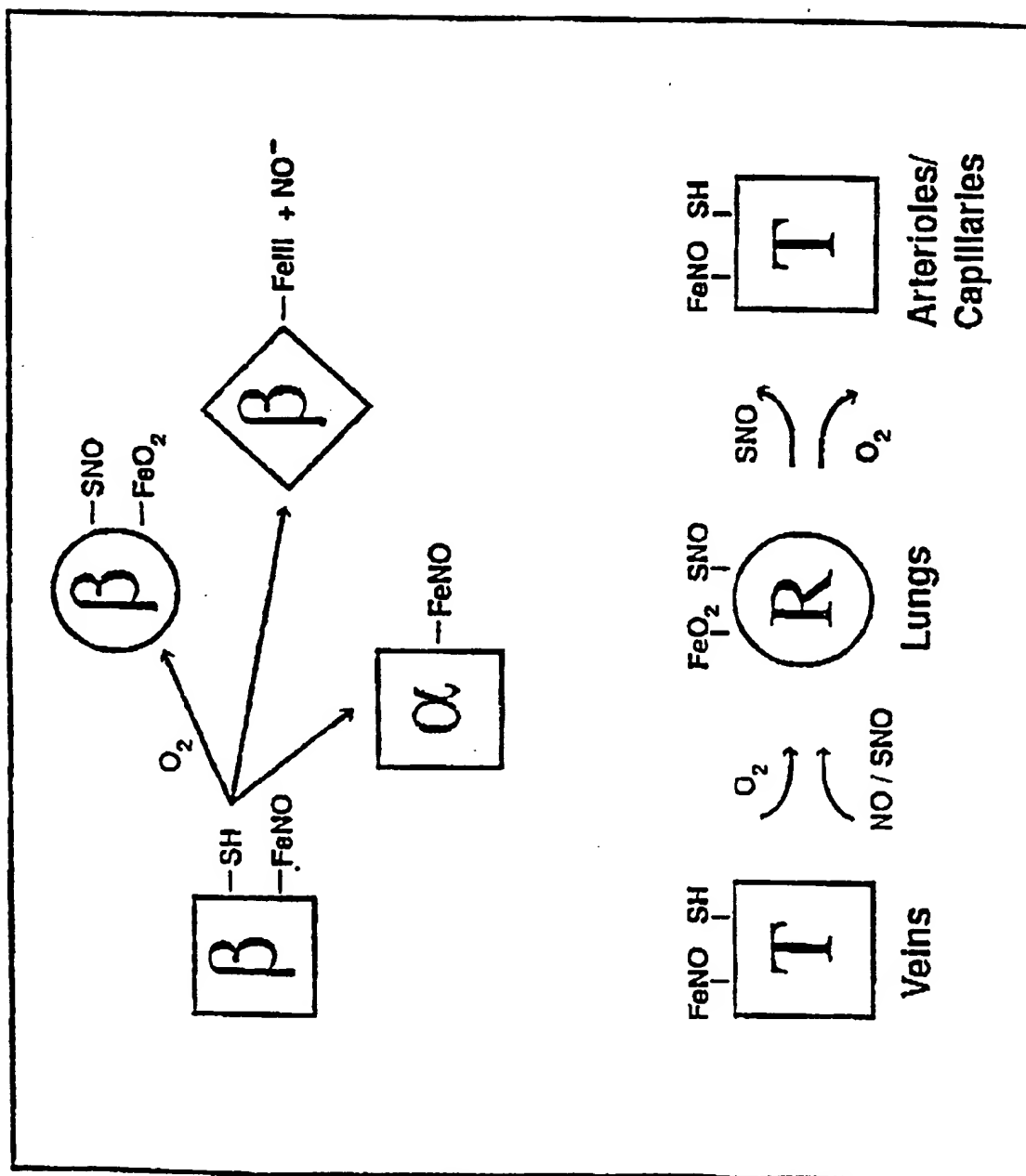


Fig. 26

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 98/02383

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 6 C07K14/805 A61K47/48 A61K38/42 G01N33/72		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 6 C07K A61K G01N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 05397 A (JEN-CHANG HSIA) 23 February 1995 see claims 26-28 ---	1,6
X	WO 93 09806 A (BRIGHAM & WOMEN'S HOSPITAL HOSPITAL) 27 May 1993 see claims 18-22 ---	1,2,6
X	WO 96 30006 A (BRIGHAM & WOMEN'S HOSPITAL) 3 October 1996 see example 20 --- -/--	1,2,6,9
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "A" document member of the same patent family		
Date of the actual completion of the international search  8 June 1998		Date of mailing of the international search report  24 06. 1998
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3018		Authorized officer  Masturzo, P



# INTERNATIONAL SEARCH REPORT

Inter. Application No

PCT/US 98/02383

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LI JIA ET AL.: "S-nitrosohemoglobin; a dynamic activity of blood involved in vascular control" NATURE., vol. 391, 21 March 1996, LONDON GB, pages 221-226, XP002067324 see the whole document ---	1,2,17, 18
X	BIOLOGICAL ABSTRACTS, vol. 90, 1990 Philadelphia, PA, US; abstract no. 90068162, T P DAVIS ET AL.: "Peptide fragments derived from the beta-chain of hemoglobin (hemorphine) are centrally active in vitro" XP002067326 & PEPTIDES, vol. 10, no. 4, 1989, pages 747-751, see abstract ---	21
P,X	WO 97 10265 A (DUKE UNIVERSITY) 20 March 1997 see the whole document ---	1,2
P,X	A J GOW ET AL.: "Reactions between nitric oxide and hemoglobin under physiological conditions" NATURE., vol. 391, 8 January 1998, LONDON GB, pages 169-173, XP002067325 see the whole document -----	1-32

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

Although claims 1-2,9-11, 20-21 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Although claims 31-32 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-8, 28-32

Method to treat or prevent a disease by administration of heme containing donors of NO, hemoglobin conjugated to NO donors, methods for the preparation of these hemoglobin derivatives and analytical methods directed to dosage of these hemoglobin-N derivatives

2. Claim : 9

Method to treat pathologies characterized by heme administration and inhalation of NO

3. Claims: 10-11

A method to deliver CO in pathological conditions

4. Claims: 12-17

Nitrosylhemoglobin coupled to electron acceptors

5. Claims: 18-19

Manufacture of erythrocytes comprising nitrosylhemoglobin

6. Claim : 20

Method of treatment of shock by administration of hemoglobin alpha-chain

7. Claim : 21

A method to treat a pathology by administration of hemoglobin beta-chain

8. Claims: 22-27

Methods to measure NO in various forms in red blood cells

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/02383

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9505397 A	23-02-1995	US 5591710 A	07-01-1997
		AT 163186 T	15-02-1998
		CA 2169178 A	23-02-1995
		CN 1133050 A	09-10-1996
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		WO 9710493 A	20-03-1997
		WO 9709972 A	20-03-1997

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-8, 28-32

Method to treat or prevent a disease by administration of heme containing donors of NO, hemoglobin conjugated to NO donors, methods for the preparation of these hemoglobin derivatives and analytical methods directed to dosage of these hemoglobin-N derivatives

2. Claim : 9

Method to treat pathologies characterized by heme administration and inhalation of NO

3. Claims: 10-11

A method to deliver CO in pathological conditions

4. Claims: 12-17

Nitrosylhemoglobin coupled to electron acceptors

5. Claims: 18-19

Manufacture of erythrocytes comprising nitrosylhemoglobin

6. Claim : 20

Method of treatment of shock by administration of hemoglobin alpha-chain

7. Claim : 21

A method to treat a pathology by administration of hemoglobin beta-chain

8. Claims: 22-27

Methods to measure NO in various forms in red blood cells

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/02383

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9505397 A	23-02-1995	US 5591710 A AT 163186 T CA 2169178 A CN 1133050 A DE 69408539 D EP 0714407 A EP 0770627 A JP 9501681 T US 5725839 A US 5741893 A	07-01-1997 15-02-1998 23-02-1995 09-10-1996 19-03-1998 05-06-1996 02-05-1997 18-02-1997 10-03-1998 21-04-1998
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L Number	Hits	Search Text	DB	Time stamp
-	12813	bilirubin carotene lycopene lutein retinoid caratenoid plastigquinone melatonin allopurinol oxypurinol methylprednisolone lazaroids tirilazad norphenazone	USPAT; US-PGPUB	2003/04/17 22:29
-	1603	(bilirubin carotene lycopene lutein retinoid caratenoid plastigquinone melatonin allopurinol oxypurinol methylprednisolone lazaroids tirilazad norphenazone) and (hemoglobin or heme-albumin)	USPAT; US-PGPUB	2003/04/17 16:44
-	1081	((bilirubin carotene lycopene lutein retinoid caratenoid plastigquinone melatonin allopurinol oxypurinol methylprednisolone lazaroids tirilazad norphenazone) and (hemoglobin or heme-albumin)) and bilirubin	USPAT; US-PGPUB	2003/04/17 16:49
-	1081	hemoglobin and bilirubin	USPAT; US-PGPUB	2003/04/17 16:45
-	640	(hemoglobin and bilirubin) and (conjugate or complex)	USPAT; US-PGPUB	2003/04/17 17:05
-	606	((hemoglobin and bilirubin) and (conjugate or complex)) and blood	USPAT; US-PGPUB	2003/04/17 17:05
-	85	((hemoglobin and bilirubin) and (conjugate or complex)) and blood and antioxidant	USPAT; US-PGPUB	2003/04/17 17:11
-	0	blood adj substitute4	USPAT; US-PGPUB	2003/04/17 17:11
-	760	blood adj substitute	USPAT; US-PGPUB	2003/04/17 17:11
-	343	(blood adj substitute) not hemoglobin	USPAT; US-PGPUB	2003/04/17 17:14
-	253	((blood adj substitute) not hemoglobin) and oxygen	USPAT; US-PGPUB	2003/04/17 17:19
-	1248	oxygen adj (transporting or carrying)	USPAT; US-PGPUB	2003/04/17 17:19
-	663	(oxygen adj (transporting or carrying)) not hemoglobin	USPAT; US-PGPUB	2003/04/17 17:20
-	81	((oxygen adj (transporting or carrying)) not hemoglobin) and (blood adj substitute)	USPAT; US-PGPUB	2003/04/17 17:28
-	208	fluorocarbon and (bilirubin carotene lycopene lutein retinoid caratenoid plastigquinone melatonin allopurinol oxypurinol methylprednisolone lazaroids tirilazad norphenazone)	USPAT; US-PGPUB	2003/04/17 17:35
-	156	(fluorocarbon and (bilirubin carotene lycopene lutein retinoid caratenoid plastigquinone melatonin allopurinol oxypurinol methylprednisolone lazaroids tirilazad norphenazone)) and (conjugate or conjugated or cross-linked or linked or complex)	USPAT; US-PGPUB	2003/04/17 21:41
-	1324	fluorocarbon and perfluorocarbon	USPAT; US-PGPUB	2003/04/17 17:35
-	253	(fluorocarbon and perfluorocarbon) and (blood adj substitute)	USPAT; US-PGPUB	2003/04/17 17:36
-	50	((fluorocarbon and perfluorocarbon) and (blood adj substitute)) and antioxidant	USPAT; US-PGPUB	2003/04/17 17:36
-	39	((fluorocarbon and perfluorocarbon) and (blood adj substitute)) and antioxidant) and (conjugate or conjugated or cross-linked or linked or complex or bound)	USPAT; US-PGPUB	2003/04/17 21:15
-	1054	perfluorocarbon and (conjugate or conjugated or cross-linked or linked or bound)	USPAT; US-PGPUB	2003/04/17 17:52

-	537	(perfluorocarbon and (conjugate or conjugated or cross-linked or linked or bound)) and blood	USPAT; US-PGPUB	2003/04/17 22:16
-	403	((perfluorocarbon and (conjugate or conjugated or cross-linked or linked or bound)) and blood) and oxygen	USPAT; US-PGPUB	2003/04/17 17:54
-	2	perfluorocarbon and trolox	USPAT; US-PGPUB	2003/04/17 17:52
-	71	((perfluorocarbon and (conjugate or conjugated or cross-linked or linked or bound)) and blood) and oxygen) and (bilirubin carotene lycopene lutein retinoid caratenoid plastigquinone melatonin allopurinol oxypurinol methylprednisolone lazaroids tirilazad norphenazone)	USPAT; US-PGPUB	2003/04/17 18:16
-	0	non-hemoglobin adj2 blood adj substitute	USPAT; US-PGPUB	2003/04/17 18:16
-	11	non-hemoglobin adj2 blood	USPAT; US-PGPUB	2003/04/17 20:49
-	84	mutant adj hemoglobin	USPAT; US-PGPUB	2003/04/17 20:49
-	151	(fluorocarbon and perfluorocarbon) and (blood adj substitute)	USPAT; US-PGPUB	2003/04/17 21:12
-	11	((fluorocarbon and perfluorocarbon) and (blood adj substitute)) and antioxidant) and (conjugate or coupled or conjugated or cross-linked or linked or complex or bound)	USPAT; US-PGPUB	2003/04/17 21:16
-	35945	fluorocarbon or perfluorocarbon or hemoglobin	USPAT; US-PGPUB	2003/04/17 21:41
-	2357	(fluorocarbon or perfluorocarbon or hemoglobin) and acetonitrile	USPAT; US-PGPUB	2003/04/17 21:41
-	359	(bilirubin carotene lycopene lutein retinoid caratenoid plastigquinone melatonin allopurinol oxypurinol methylprednisolone lazaroids tirilazad norphenazone antioxidant) and ((fluorocarbon or perfluorocarbon or hemoglobin) and acetonitrile)	USPAT; US-PGPUB	2003/04/17 22:14
-	9712	myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin	USPAT; US-PGPUB	2003/04/17 22:17
-	9238	(conjugate or conjugated or cross-linked or linked or bound or bound or coupled) and (myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin)	USPAT; US-PGPUB	2003/04/17 22:18
-	7775	( (conjugate or conjugated or cross-linked or linked or bound or bound or coupled) and (myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin)) and blood	USPAT; US-PGPUB	2003/04/17 22:18
-	2230	( (conjugate or conjugated or cross-linked or linked or bound or bound or coupled) and (myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin)) and acetonitrile	USPAT; US-PGPUB	2003/04/17 22:17
-	139	( (conjugate or conjugated or cross-linked or linked or bound or bound or coupled) and (myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin)) and (blood adj substitute)	USPAT; US-PGPUB	2003/04/17 22:18
-	32	(bilirubin carotene lycopene lutein retinoid caratenoid plastigquinone melatonin allopurinol oxypurinol methylprednisolone lazaroids tirilazad norphenazone antioxidant) and ((conjugate or conjugated or cross-linked or linked or bound or bound or coupled) and (myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin)) and (blood adj substitute))	USPAT; US-PGPUB	2003/04/17 22:29



-	47	(bilirubin carotene lycopene lutein retinoid caratenoid plastigquinone melatonin allopurinol oxypurinol methylprednisolone lazaroids tirilazad norphenazone antioxidant) and ((conjugate or conjugated or cross-linked or linked or bound or bound or coupled) and (myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin)) and (blood adj substitute))	USPAT; US-PGPUB	2003/04/17 23:08
-	69	(phenolic or pyrazoline or carotenoid or retinoid or quinone or tetrapyrrole or indole or aminoindole or purine or ascorbic steroid) and ((conjugate or conjugated or cross-linked or linked or bound or bound or coupled) and (myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin)) and (blood adj substitute))	USPAT; US-PGPUB	2003/04/17 23:08
-	2125	(bilirubin carotene lycopene lutein retinoid caratenoid plastigquinone melatonin allopurinol oxypurinol methylprednisolone lazaroids tirilazad norphenazone antioxidant) and (myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin)	USPAT; US-PGPUB	2003/04/17 23:08
-	4850	(phenolic or pyrazoline or carotenoid or retinoid or quinone or tetrapyrrole or indole or aminoindole or purine or ascorbic steroid) and (myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin)	USPAT; US-PGPUB	2003/04/17 23:28
-	69	((phenolic or pyrazoline or carotenoid or retinoid or quinone or tetrapyrrole or indole or aminoindole or purine or ascorbic steroid) and ((conjugate or conjugated or cross-linked or linked or bound or bound or coupled) and (myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin)) and (blood adj substitute))) and ((phenolic or pyrazoline or carotenoid or retinoid or quinone or tetrapyrrole or indole or aminoindole or purine or ascorbic steroid) and (myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin))	USPAT; US-PGPUB	2003/04/17 23:09
-	1	chromanol and (myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin)	USPAT; US-PGPUB	2003/04/17 23:29
-	438	(catechol or phenolic or dihydrobenzofuranol or isoflavanoid or flavanoid) and (myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin)	USPAT; US-PGPUB	2003/04/17 23:38
-	52	intromolecular	USPAT; US-PGPUB	2003/04/17 23:38
-	14692	intramolecular	USPAT; US-PGPUB	2003/04/17 23:38
-	1547	intramolecular and ((myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin))	USPAT; US-PGPUB	2003/04/17 23:39
-	201	(intramolecular and ((myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin))) and (heme or hemoglobin)	USPAT; US-PGPUB	2003/04/17 23:39

-	90	((intramolecular and ((myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin))) and (heme or hemoglobin)) and ((bilirubin carotene lycopene lutein retinoid caratenoid plastigquinone melatonin allopurinol oxypurinol methylprednisolone lazaroids tirilazad norphenazone antioxidant) and (myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin))	USPAT; US-PGPUB	2003/04/17 23:41
-	1643	(myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin) and (trolox or ascorbic or chromanol)	USPAT; US-PGPUB	2003/04/17 23:42
-	20	(myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin) and (trolox or chromanol)	USPAT; US-PGPUB	2003/04/17 23:45
-	9238	(myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin) and ( (conjugate or conjugated or cross-linked or linked or bound or bound or coupled) and (myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin))	USPAT; US-PGPUB	2003/04/17 23:45
-	1	((myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin) and ( (conjugate or conjugated or cross-linked or linked or bound or bound or coupled) and (myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin))) and chromanol	USPAT; US-PGPUB	2003/04/17 23:45
-	19	((myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin) and ( (conjugate or conjugated or cross-linked or linked or bound or bound or coupled) and (myoglobin or hemrythrin or hemocyanin or carbonmonoxyhemoglobin))) and trolox	USPAT; US-PGPUB	2003/04/17 23:45